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CONTENTS

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CANCER RESEARCH

VOLUME 11 MARCH 1951 NUMBER 3

The Effect of Carcinogenic and Other Related Compounds on the Autoxidation of Carotene and Other Autoxidizable Systems

E. B. LISLE

(From 106, Durham Road, Blackhill Co., Durham, Eng.)

Several investigators have shown that certain carcinogenic chemicals and polycyclic hydrocarbons inhibit the autoxidation of benzaldehyde and lipoidal materials (5–7). This effect on benzaldehyde was confirmed. Further experiments carried out with carotene in solvents containing carbon tetrachloride or benzoyl peroxide showed that, under the influence of light, many carcinogens and some other hydrocarbons caused an increase in the rate of oxidation of the carotene. The results compared well with those obtained in another photochemical method in which certain leuco dyestuffs, in the presence of silver bromide, were dehydrogenated very rapidly under illumination, when treated with carcinogens (4).

MATERIALS AND METHODS

The autoxidation of benzaldehyde.—An indigo-benzaldehyde solution was prepared by diluting 10 ml. of redistilled benzaldehyde to 50 ml. with ethyl alcohol containing 1.5 ml. of a 0.05 per cent aqueous solution of indigo carmine. Five-ml. portions of the solution were placed in several tubes along with 0.2 ml. of the various compounds under test in acetone (0.025 per cent). A blank test was carried out simultaneously, and the reaction was allowed to proceed in darkness at room temperature. The fading was followed using the "Hilger Biochemical Absorptiometer" (Table 1).

The autoxidation of carotene.—A carotene solution was prepared by extracting dried carrots with petroleum ether (60°–80° C.), and the solution was adjusted to the color of an 0.02 N potassium dichromate solution. Standard dichromate solutions were also prepared equivalent to 50, 25, and 12.5 per cent of the concentration of the original solution. A solution of carotene in acetone was also prepared by evaporating the petroleum ether and by dissolving the residue in acetone. For comparative purposes, solutions were made using a pure sample of carotene, supplied by "British Drug Houses Ltd." England, containing 10 per cent α and 90 per cent β isomers. Three-ml. portions of the standard carotene solution in petroleum ether, each containing 1 ml. of carbon tetrachloride (redistilled) were placed in a series of tubes, 1 × 10 cm., along with 0.2 ml. of a particular carcinogen in pure benzene (0.025 per cent). These polycyclic hydrocarbons were pure samples supplied by Pro-

TABLE 1

THE AUTOXIDATION OF BENZALDEHYDE AS SHOWN BY THE DECOLORIZATION OF INDIGO CARMINE

COMPOUND	No. OF TEST	PERCENTAGE DECOLORIZATION 28 hours	52 hours
Blank	1	64.5	71.8
	2	64.2	71.8
Methylcholanthrene	1	53.2	64.5
	2	52.8	64.5
Chrysene	1	60.7	69.9
	2	60.5	69.6
2,7-Dimethyl-1,2-Benzanthracene	1	57.7	69.9
	2	57.3	70.2
1,2,5,6-Dibenzanthracene	1	60.0	70.3
	2	59.7	70.2
1,2,5,6-Dibenzfluorene	1	60.7	68.6
	2	60.2	68.4
3,4-Benzphenanthrene	1	64.5	71.8
	2	64.8	71.4
Pyrene	1	54.2	64.1
	2	53.8	63.8
3,4-Benzpyrene	1	52.2	60.0
	2	52.0	60.3
Anthracene	1	57.7	67.2
	2	57.6	67.5

Received for publication May 16, 1950.

TABLE 2

THE ACTION OF CARCINOGENS AND OTHER COMPOUNDS ON THE AUTOXIDATION OF CAROTENE IN THE PRESENCE OF CARBON TETRACHLORIDE

Compound	No. of test	Petroleum ether solution: Percentage decolorization (minutes) 12.5	25	50	K Velocity	Acetone solution: Percentage decolorization (minutes) 12.5	25	50	K Velocity	Biological Activity*†
Blank	1	185	340			58	76	93		
	2	188	340			61	76	99		
	3	196	360			60	75	95		
	Av.	190	350		0.0038	60	76	96	0.0169	
Methylcholanthrene	1	24	68	89		17	33			
	2	28	73	93		21	31			
	3	29	70	88		19	27			
	Av.	27	70	90	0.0231	19	30		0.0413	A
9,10-Dimethyl-1,2-Benzanthracene	1	34	76	100		21	31	44		
	2	31	80	110		22	37	44		
	3	30	78	105		22	37	45		
	Av.	32	78	105	0.0200	22	35	45	0.0396	A
3,4-Benzpyrene	1	22	54	80		22	28	35		
	2	26	56	88		21	33	45		
	3	26	53	83		22	31	41		
	Av.	25	55	84	0.0248	22	31	40	0.0428	A
1,2,5,6-Dibenzanthracene	1	185				57	72	99		
	2	180				61	77	93		
	3	205				63	78	95		
	Av.	190			0.0038	60	76	96	0.0169	A
3,4-Benzphenanthrene	1	115	300			63	71	94		
	2	115	315			58	77	98		
	3	130	330			61	80	97		
	Av.	120	315		0.0051	60	76	96	0.0169	B
5,6,9,10-Tetramethyl-benzanthracene	1	155	280							
	2	175	310							
	3	164	310							
	Av.	165	300		0.0084					B
1,2,5,6-Dibenzfluorene	1	34	59	92		25	45	51		
	2	33	62	97		28	38	53		
	3	34	63	95		29	37	57		
	Av.	34	62	95	0.0220	27	40	54	0.0330	C
β-Naphthylamine	1	100	230			62	100			
	2	106	260			61	111			
	3	115	244			58	107			
	Av.	110	245		0.0060	60	106		0.0120	C
Pyrene	1	73	150	240		41	57	70		
	2	73	160	255		35	55	77		
	3	78	155	255		35	51	81		
	Av.	75	155	250	0.0096	37	54	76	0.0246	D
2,7-Dimethyl-1,2-Benzanthracene	1	31	74	100		22	30	42		
	2	35	75	100		22	30	38		
	3	36	75	100		27	29	37		
	Av.	34	75	100	0.0208	24	30	39	0.0428	D
Anthracene	1	25	37	65		17	21	36		
	2	24	42	70		15	26	34		
	3	26	40	66		11	25	37		
	Av.	25	40	67	0.0310	14	24	36	0.0530	D
Chrysene	1	195	320			44	66			
	2	180	360			43	56			
	3	180	325			43	61			
	Av.	185	335		0.0035	43	60		0.0145	D
1,2-Benzanthracene	1	48	150	280						
	2	47	170	310						
	3	55	167	300						
	Av.	50	162	290	0.0098					D
Cholesterol	1	190								
	2	190								
	3	190								
	Av.	190			0.0038					D
Citral	1	over 180				over 180				D
Hydroquinone	1	over 180				over 180				D
Ascorbin	1							96		D

* A = strongly carcinogenic; B = weakly carcinogenic; C = moderately carcinogenic; D = noncarcinogenic.
† Commercially pure carotene used in No. 3 test.

fessor J. W. Cook of Glasgow University, and fresh solutions were prepared for each series of tests to insure that oxidation to quinones did not occur. The tubes and contents were then illuminated by means of a 100-watt metal filament lamp, at a distance of 10 cm. A blank test was carried out under similar conditions, and the rates of fading were followed with the prepared color standards. The tests were repeated in an acetone solution of carotene, the carcinogens being dissolved in that solvent also. Finally, the tests were again repeated using a 0.4 per cent solution of benzoyl peroxide in ethyl acetate in place of the carbon tetrachloride.

Silver bromide reaction (4).—Strips of filter paper were prepared by soaking them in a 4 per cent aqueous solution of silver nitrate containing either 5 ml. of a 0.08 per cent aqueous solution of leucomethylene blue or leucomalachite green, and then in a 1.8 per cent potassium bromide solution. After drying in darkness, the papers were spotted with a benzene solution of the particular compound and illuminated with light of wave length 400–450 mμ. A blue or green spot indicated a positive reaction.

RESULTS

The autoxidation of benzaldehyde.—In the presence of indigo the autoxidation of benzaldehyde can be followed, since the oxidation of the dye to faintly colored oxidation products runs parallel to the oxidation of the aldehyde to benzoic acid. The oxidation is due to the oxidizing action of the intermediate, benzoyl hydrogen peroxide, and reproducible results can be obtained (Table 1). It will be noted that the powerful carcinogens methylcholanthrene and 3,4-benzpyrene (1) act as moderately strong anti-oxidants on the system, while noncarcinogenic anthracene, chrysene, and 2,7-dimethyl-1,2-benzanthracene (1) act as weaker anti-oxidants. Noncarcinogenic pyrene, however, acts in a moderately strong manner, and the powerful carcinogen 1,2,5,6-dibenzanthracene has only a weak action.

Carotene oxidation systems.—Under the influence of light, carotene in acetone or petroleum ether solution containing carbon tetrachloride became decolorized. The final solution was slightly acid and was found to contain oxidation products of carotene—e.g., β-ionone was present, indicating the breakdown of the carotene chain. It is suggested that, under the influence of light, free trichloromethyl radicals (3) combine with the unsaturated hydrocarbon carotene and then, under the influence of molecular oxygen, an autoxidation process takes place, the final products of the reaction containing colorless oxidation products of carotene. There is some evidence to support this view, as an experiment was performed in which oxygen was rigorously excluded from the system, and the reaction was carried out under oxygen-free nitrogen. Under these conditions, no decolorization of the carotene solution took place, and, indeed, there was a slight darkening of the yellow solutions. On readmitting oxygen, normal oxida-

TABLE 3

THE ACTION OF CARCINOGENS AND OTHER COMPOUNDS ON THE AUTOXIDATION OF CAROTENE IN THE PRESENCE OF BENZOYL PEROXIDE

Compound	No. of test	Time in minutes 50 per cent decolorization
Blank	1	87
	2	95
Methylcholanthrene	1	50
	2	45
1,2,5,6-Dibenzanthracene	1	45
	2	42
2,7-Dimethyl-1,2-Benzanthracene	1	46
	2	40
3,4-Benzphenanthrene	1	45
	2	40
1,2,5,6-Dibenzfluorene	1	41
	2	48
3,4-Benzpyrene	1	44
	2	50
Pyrene	1	38
	2	46
Chrysene	1	56
	2	48
Anthracene	1	40
	2	40
9,10-Dimethyl-1,2-Benzanthracene	1	56
	2	50
Cholesterol	1	90
	2	85
Hydroquinone		over 100
Pyrogallic acid		over 100
β-Naphthylamine	1	92
	2	88

TABLE 4

THE ACTION OF CARCINOGENS ON SILVER BROMIDE LEUCO DYESTUFF TEST PAPERS

Compound	Depth* of color
Methylcholanthrene	100
Cholanthrene	90–100
3,4-Benzpyrene	100
1,2-Benzanthracene	0
9,10-Dimethyl-1,2,5,6-Dibenzanthracene	0
3,4-Benzphenanthrene	0
5,6,9,10-Tetramethyl-1,2-Benzanthracene	50–75
1,2,5,6-Dibenzfluorene	50–75
2,7-Dimethyl-1,2-Benzanthracene	0
Anthracene	0–10
Chrysene	0
Pyrene	80–100
1,2,5,6-Dibenzanthracene	0
9,10-Dimethyl-1,2-Benzanthracene	50–75

*Methylcholanthrene given arbitrary value of 100. Comparative measurements made using a photoelectric cell.

tion again took place, the reaction being photochemical, only taking place under the action of light. The presence of added carcinogens and allied compounds greatly increased the reaction rate, indicating that these compounds acted as pro-oxidants on the system. This reaction appeared to approximate a "First order reaction," and the velocity coefficients K were calculated (Table 2). Trichloroacetic acid acted similarly to carbon tetrachloride, but chloroform had no visible action. Benzoyl peroxide acted positively, the final products of the reaction being benzoic acid and carotene oxidation products (Table 3). A combination with free phenyl and benzoyl radicals (2, 3), formed from the diaroyl peroxide, with carotene, may initiate an autoxidation process as in the carbon tetrachloride reaction. When a purified sample of carotene in acetone solution was substituted from the crude extract, comparable results were obtained, impurities present having little effect on the course of the reaction.

Silver bromide reaction.—The action of some carcinogens on silver bromide dye paper, under the influence of light, appears to be another example of the pro-oxidant effect of these compounds, as the dye in this case was oxidized and consequently deeply colored (Table 4).

DISCUSSION

An examination of the results obtained in all four systems seems to indicate some correlation between oxidation rates and biological activity. In the benzaldehyde system the inhibition of oxidation seems to run parallel with high biological activity (Table 1), while in the carotene systems an increasing rate of oxidation follows an increase in the potency of the carcinogen. The depth of color of the spot obtained in the silver bromide reaction is in many cases a rough measure of the activity of the compound being tested. Table 2 shows that the reaction velocity is much greater in acetone solution than in petroleum ether, but otherwise the results compare moderately well. There is a deviation in the case of β-naphthylamine, which has been shown to be carcinogenic (1), the compound causing an increased reaction velocity in ether solution, as compared to a blank. This does not occur in acetone solution. The reactions are not absolutely specific for carcinogenic compounds in any of the four systems, as 1,2,5,6-dibenzanthracene does not increase the reaction velocity in the carotene-carbon tetrachloride system or decrease the velocity in the benzaldehyde reaction, nor does it give a positive result with silver bromide. 2,7-Dimethyl-1,2-benzanthracene, anthracene, and pyrene give a marked velocity increase in carotene systems, but in the silver bromide test, 2,7-dimethyl-1,2-benzanthracene gives a negative reaction and anthracene a slightly positive one. With benzaldehyde these two compounds show only moderately weak anti-oxidant activity. The benzoyl peroxide-carotene reaction is not very specific for carcinogens, as many noncarcinogens show a positive reaction, but here 1,2,5,6-dibenzanthracene acts positively. On considering the four systems generally, it seems evident that the carcinogenic compounds tested can act as catalysts on autoxidizing systems by increasing or decreasing the rate of oxidation, and it is suggested that there might be some systems of enzymes in animal tissues on which the known carcinogens act specifically as pro-oxidants, and others on which they act as anti-oxidants.

SUMMARY

The inhibition of the autoxidation of benzaldehyde by carcinogenic and other polycyclic hydrocarbons was confirmed, the more powerful carcinogens showing the greater effect. Under the influence of light, carotene in solvents in the presence of carbon tetrachloride or benzoyl peroxide became oxidized, an effect which was accelerated by many carcinogenic compounds. These results compared fairly well with those obtained when a silver bromide leuco dyestuff-impregnated filter paper was illuminated in the presence of a carcinogen, a colored spot appearing in many cases. It was suggested that the carcinogens might either act as pro-oxidants or anti-oxidants on specific animal enzyme systems.

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An Evaluation of the Bolen Test as a Screening Test for Malignancy*

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Bolen (2) in 1942 reported the blood droplet pattern as distinctive in cancer and proposed it as a valuable aid in the diagnosis of the disease. He found the test to be accurate in 91.4 per cent of all cancer patients studied. Giron (5) in 1943, using the method of Bolen, obtained similar results.

In our study the test was performed on 857 individuals. First, a preliminary study was performed on 195 healthy subjects and on 157 patients with histologically proved cancer, to familiarize us with features of the normal or negative and positive blood patterns.

Grossly, the normal pattern (Fig. 1) is a uniform, compact design with the central portion showing a dense, dark spot or nucleus. The pattern associated with cancer is different in most cases (Fig. 2). There is a breaking-down of the compact design near the mid-central part of the drop, and the central nucleus or spot becomes less distinct or completely absent. In advanced cancer, the appearance is that of a "dotted curtain" (Fig. 3).

Microscopically, the normal pattern (Fig. 4) reveals a well defined weblike fibrin meshwork extending over the entire drop. There are occasional leukocytes and normal appearing, tightly packed red cells with rouleau formation.

In cancer (Figs. 5 and 6), the fibrin meshwork is absent, and only small fibrin threads are seen. There are many small plasma lakes throughout the drop. The plasma lakes are clear, vary in size, and are devoid of erythrocytes. The red cells appear in clumps, are irregular in outline and shape, and rouleau formation is absent.

A normal or negative blood pattern was found in all 195 healthy individuals, and a positive pattern was found in 87 per cent of the 157 patients with a histologically proved cancer. No attempt was made to select the cancer patients as to site or extent of the disease (Table 1).

These findings are comparable to the results found in the literature (Table 2), except for a small series (25 patients) reported by us with Finnegan (4), in which a positive pattern was found in only 13 patients.

We then studied 505 successive new patients who entered the diagnostic and cancer-detection clinic at Firmin Desloge Hospital of St. Louis University from July, 1949, to October, 1949. This group included patients with a variety of diagnoses, and the test was performed and interpreted on each patient without any knowledge of the clinical history or findings. The test was applied to this particular group as a screening test.

TABLE 1

BLOOD PATTERN IN NORMAL AND CANCER PATIENTS

Subjects	Blood Pattern		Total	Per cent positive
	Positive	Negative		
Normal	0	195	195	0
Cancer (histologically proved)	140	17	157	87

TABLE 2

RESULTS REPORTED IN THE LITERATURE OF THE BLOOD PATTERN IN MALIGNANCY

Year	Author	No. cancer patients	Per cent positive pattern
1942	Bolen (2)	140	91
1943	Giron (5)	150	90
1943	Coltman (3)	35	97
1950	Inkley *et al.* (6)	100	88
1950	Finnegan *et al.* (4)	25	53
1950	Black and Speer (1)	100	80
1950	Present paper	157	87

METHOD[1]

The patient's fingertip is cleansed with ether or alcohol, allowed to dry, pricked with a lancet, and a free flow of blood obtained. Six or seven drops, varying in size and thickness, are collected on a slide by lightly touching the drop present on the fingertip, being careful not to touch the finger with the slide. This may interfere with the formation of the pattern. The slide is placed on a flat surface and allowed to stand until the drops are dry. The pattern formed in each drop is examined both

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[1] As described by Bolen, in 1942, with a few minor variations.

macroscopically and microscopically at the examiner's convenience.

RESULTS

Analysis of the 505 clinic patients, studied without reference to the clinical findings, revealed 439 patients with a negative or normal blood pattern and 66 with a positive blood pattern (Table 3).

TABLE 3

RESULTS OF THE BOLEN TEST ON 505 SUCCESSIVE NEW PATIENTS FROM THE DIAGNOSTIC CLINIC*

Diagnoses (grouped)	Positive	Negative	Total patients
Pregnancy	36	46	82
Neoplasms			
Benign	0	9	9
Malignant	12	2	14
Heart disease	2	27	29
Ear, eye, nose, throat diseases	2	71	73
Gastro-intestinal disease (benign)	2	40	42
Dermatitis	2	12	14
Tuberculosis, pulmonary	1	6	7
Pelvic inflammatory disease	2	58	60
Arthritis (degenerative)	2	11	13
Syphilis	1	19	20
Genito-urinary disease	1	20	21
Neuro-psychiatric disease	1	22	23
Menopausal syndrome	1	9	10
Pulmonary disease (not tuberculosis or tumor)	1	19	20
Essential hypertension	0	10	10
Thyroid disease	0	12	12
Dietary deficiency	0	5	5
Arteriosclerosis (general)	0	28	28
Diabetes mellitus	0	3	3
No disease found	0	5	5
Skeletal diseases (fractures)	0	2	2
Peripheral vascular diseases	0	3	3
Totals	66	439	505

* Children 14 years of age and younger excluded from this study.

There were 14 malignant neoplasms found in these 505 patients. Twelve of the 14 patients showed a positive pattern, while the remaining 2 patients showed a negative pattern. One of the negative patterns was found in a case of far advanced adenocarcinoma of the breast and the other in a patient with bronchogenic carcinoma with metastases. The droplet pattern in subsequent examinations was constantly negative in these two cases. The remaining 54 patients with a positive blood pattern did not have cancer, and all except those pregnant were re-examined at regular intervals for 1 year after treatment. The blood pattern returned to normal in all but 1 patient (Table 4).

There were 2 false negative and 54 false positive tests found in this group of 505 patients.

DISCUSSION

The fourteen neoplasms found in the screening group are divided into two groups: those in patients having an obvious cancer and in those having a "hidden" cancer—that is, one which is not readily identified by the common clinical examinations (Table 5).

In ten of the fourteen cases the clear-cut diagnosis of cancer was made without difficulty by the conventional clinical examination. The blood pattern was found to be positive in eight of these patients, who were shown to have the following lesions: squamous-cell carcinoma of the tongue with cervical metastases, adenocarcinoma of the stomach, squamous-cell carcinoma of the skin (4), squamous-cell carcinoma of the tongue, and adenocarcinoma of the rectum. The blood pattern was negative in two of these ten patients—one having an adenocarcinoma of the breast, with axillary and pulmonary metastases, and the other a far advanced bronchogenic carcinoma with cervical metastases.

A positive blood pattern was found in all four of the patients with neoplasms which were not readily detectable by the usual clinical diagnostic procedures. Thus, a persistently positive blood pattern provoked further scrutiny in these cases to establish a diagnosis of cancer as will be shown in the following case presentations.

TABLE 4

RESULTS OF RE-EXAMINATION OF PATIENTS SHOWING FALSE POSITIVE BOLEN TEST, BEFORE AND AFTER TREATMENT

Diagnosis	First examination	Positive pattern after 4 months or more
Pregnancy	36	(Not followed)
Rheumatic heart disease	2	0
Pelvic inflammatory disease	2	0*
Acute appendicitis	1	0
Common duct obstruction	1	0
Dermatitis	2	0
Acute epididymitis	1	0
Pulmonary tuberculosis	2	0*
Neuro-psychiatry	2	0
Acute pan-sinusitis	2	0
Syphilis, tertiary	1	1
Arthritis (degenerative)	2	0
Totals	54	1

* Only one case re-examined.

TABLE 5

VALUE OF THE BOLEN TEST IN CANCER

Neoplasm	No.	Bolen test	
		Positive	Negative
Obvious cancer	10	8	2
"Hidden" cancer	4	4	

There were 14 histologically proved cancers found in the 505 patients examined in the medical diagnostic and cancer detection clinics.

CASE 1

Carcinoma of the prostate gland.—J. R., a 68-year-old white man, entered the clinic August 23, 1949; the Bolen test was positive. He complained

of pain in his left eye. He was diagnosed and treated for a corneal ulcer. The repeat Bolen test was positive on September 10, 1949. Further examination revealed a large, nodular prostate gland, not thought malignant. August 18, 1950, he reentered the hospital, complaining of nausea, vomiting, and epigastric pain. Physical examination revealed tenderness in the epigastrium and a hard nodular prostate. The pathological diagnosis of the prostate gland was adenocarcinoma. The gastric lesion is thought to be a benign ulcer and is still under observation. (#49–8093, F.D.H.)

Comment.—Because the Bolen test remained positive for a year, the diagnosis of carcinoma was suggested in this patient. He originally reported with a corneal ulcer and a nodular prostate which the urologist thought benign. After a year prostatectomy revealed carcinoma present in the prostate.

CASE 2

Bronchogenic carcinoma.—R. B., a 63-year-old white man, entered the clinic July 15, 1949; the Bolen test was positive. He complained of chest pain, cough, 10 pounds' weight loss, and gave a history of tuberculosis. Physical and roentgenologic examinations resulted in a diagnosis of tuberculosis. On August 23, 1949, the Bolen test was positive. He was admitted to a sanatorium for treatment. A diagnosis of tuberculosis was not confirmed, and after exhaustive diagnostic studies, an exploratory thoracotomy was suggested. The Bolen test remained positive upon repeated examinations.

On November 12, 1949, a right pneumonectomy was performed, with an operative diagnosis of chronic abscess of right lung. The pathological report was bronchiectasis, chronic bronchitis, peribronchitis, lung abscess, and organized pneumonia. No carcinoma was seen. The post-operative course was complicated by a broncho-pleural fistula, which closed spontaneously, and the patient was discharged from the hospital January 21, 1950, greatly improved.

He was re-admitted to the hospital August 7, 1950, extremely ill with nodules in the pneumonectomy scar. Biopsy showed these to be metastatic carcinoma. He died September 26, 1950. At autopsy, bronchogenic carcinoma was found, involving the right bronchial stump, with widespread metastasis. (#49–5175, F.D.H.) Unfortunately, the surgical specimen was misplaced during the interim. Therefore, one cannot be sure whether this lesion was present in the specimen and overlooked or left behind in the right bronchial stump.

Comment.—Again, a Bolen test, positive for a year, pointed correctly to the diagnosis of carcinoma, despite clinical evidence to the contrary. Admitted as a tuberculosis suspect, the right lung of the patient was removed after 3 months for abscess, and no carcinoma found histologically—only abscess and bronchiectasis. However, after 9 months carcinoma became clinically apparent because of nodules beneath the scar of pneumonectomy, and the diagnosis was ultimately confirmed by necropsy.

CASE 3

Bronchogenic carcinoma.—E. T., a 68-year-old white woman, entered the clinic October 3, 1949; the Bolen test was positive. She complained of constant coughing, pain in the right chest, and gave a history of having been treated for pneumonia 6 weeks previously. A diagnosis of inflammatory disease of the right lung with right pleural effusion was made, and the patient was admitted to the hospital. A repeat Bolen test was positive October 10, 1949. Examination of the pleural fluid revealed malignant cells, and a bronchial biopsy confirmed the diagnosis of bronchogenic carcinoma. The Bolen test was positive on last follow-up, December 17, 1949. (#49–9702, F.D.H.)

Comment.—Here, in another patient with lung cancer, the Bolen test was positive upon admission, and the clinical diagnosis was lung abscess or unresolved pneumonia. Thoracentesis yielded fluid with cells, histologically malignant upon centrifugation.

CASE 4

Carcinoma of the left breast (male).—C. S., a 59-year-old white man, entered the clinic July 21, 1949; the Bolen test was positive. He complained of headache and a small mass just beneath the skin in the left temporal region. Roentgenologic and physical examination revealed no positive diagnosis. The Bolen test was positive again August 20, 1949. He was admitted to the hospital, and the mass was removed from the head. A diagnosis of metastatic carcinoma was made. Exhaustive tests and examinations failed to reveal the primary lesion. He died September 26, 1949, and at autopsy a very small, deep-seated, primary lesion was found in the left breast, and a diagnosis of adenocarcinoma of the breast with metastasis was made. (#49–6319, F.D.H.)

Comment.—A suspicion that the subcutaneous mass in the temporal region might be carcinoma was aroused, in this patient, by a positive Bolen test, and clinical study during the ensuing month yielded no positive data. Upon removal, the mass was then proved to be carcinoma. The primary site remained undiscovered until necropsy, when a

hidden carcinoma, primary in the (male) breast, was found.

All but 2 of the 14 patients were treated either surgically or with radium. The blood pattern returned to normal the fourth month post-operatively in a surgically treated adenocarcinoma of the rectum and has been negative upon subsequent examinations. The remainder of the treated cases continued to show a positive blood pattern.

The 54 patients showing a false positive blood pattern were re-examined and grouped as those obviously not having cancer and those suspected of having cancer (see Table 6). Thirty-six patients, all those pregnant, were not followed. Thus, of the remaining 18 patients with a false positive test, only one failed to show a normal pattern after treatment had been instituted. This was a 43-year-old white male (E. G., #49–9693, F.D.H.) complaining of pain and swelling in the right knee. There was a history of syphilis. Physical examination revealed swelling of the right knee and bloody effusion. The Bolen test was positive upon admission and at all the subsequent examinations for the past year. Exhaustive examinations including a biopsy of the right knee joint capsule failed to reveal any evidence of malignancy. To date, this case remains a false positive, and a diagnosis of tertiary syphilis with Charcot's disease stands.

The occurrence of the positive blood pattern is interpreted as a "clue" and should encourage the physician to perform exhaustive studies to exclude or prove the presence of cancer. A normal blood pattern does not exclude the possibility of cancer and should be ignored especially when there are clinical manifestations suggestive of malignancy.

SUMMARY

We have screened 505 patients for malignancy by the Bolen test. There were 66 positive tests, of which 12 were substantiated by histologic evidence of malignant neoplasm, while 54 appear to be false positives. With one exception, all the false positive tests subsequently reverted to negative.

There were 14 histologically proved cancers in the entire group of 505 patients. Two of these tumors gave repeated negative reactions to the Bolen test.

We may conclude that a negative Bolen test does not exclude the diagnosis of cancer. Nor does a positive test necessarily indicate the presence of cancer. However, a positive test should be followed by intense diagnostic effort before the diagnosis of malignant neoplasm is abandoned.

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TABLE 6

"FALSE POSITIVE" BOLEN TESTS AFTER TREATMENT AND ONE-YEAR FOLLOW-UP

Neoplasm	No.	Bolen test	
		Positive	Negative
Obviously not cancer	36 pregnancy	(Not followed)	
	17 inflammatory disease	0	15*
Suspected cancer	1	1	0

* Two lost to follow-up.

There were 54 false positive tests in 491 noncancer patients upon admission to the clinic.

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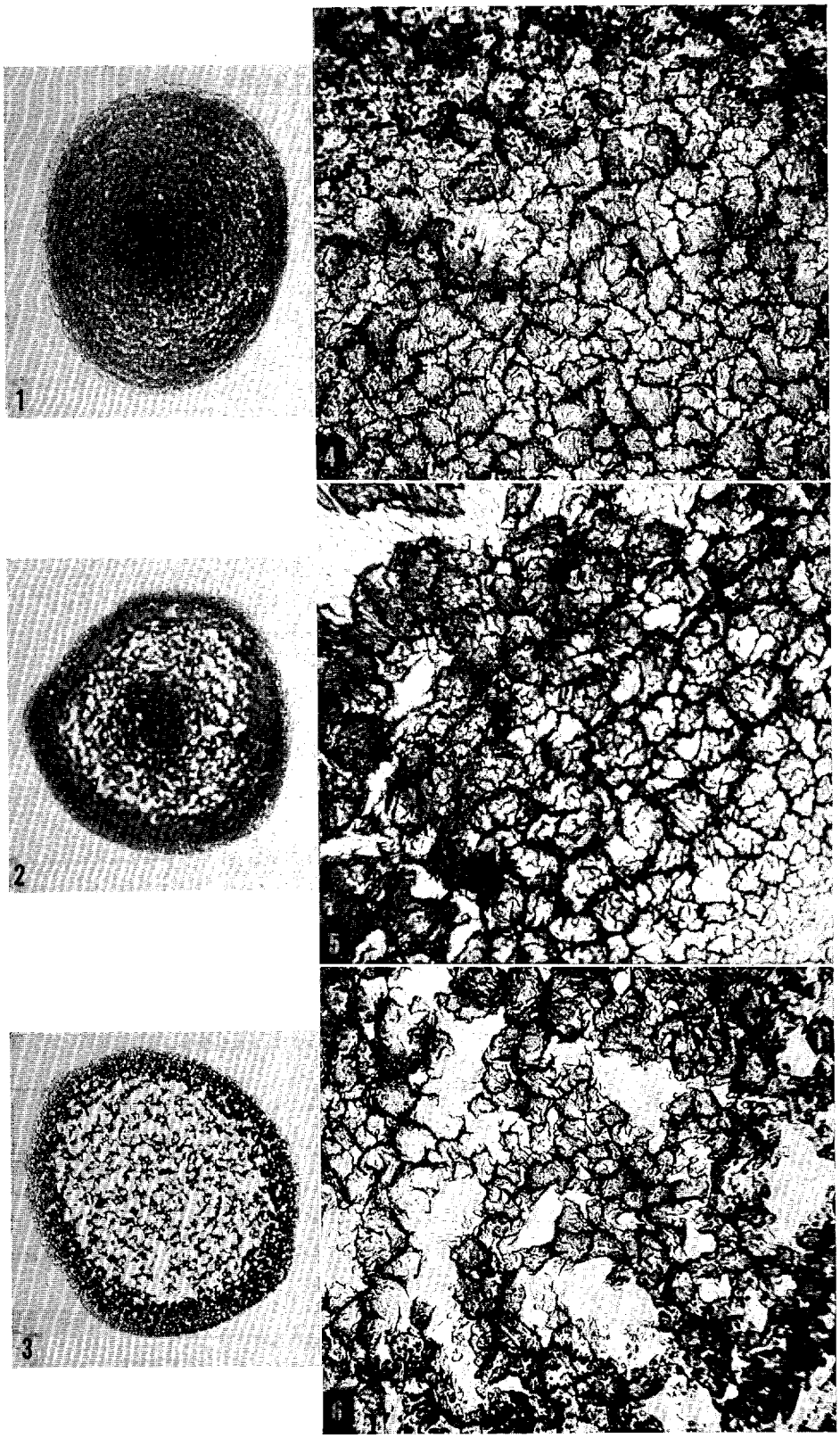
FIG. 1.—Normal blood pattern. Mag. ×5.

FIG. 2.—Blood pattern in early cancer. Mag. ×5.

FIG. 3.—Blood pattern in advanced cancer. Mag. ×5.

FIG. 4.—Microscopic view of the normal pattern taken from the central area of the drop. Mag. ×100.

FIGS. 5 and 6.—Microscopic appearance of the blood pattern in cancer as found in the central area of the drop. Mag. ×100.



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Partial Reversal of the Anti-leukemic Action of Folic Acid Antagonists by Vitamin B_{12}*

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Recently, Woolley and Pringle (12) have demonstrated the build-up of 4-amino-5-carboxamidoimidazole in *E. coli* grown in the presence of inhibiting doses of 4-aminopteroylglutamic acid (aminopterin). It is known that formate is a precursor of the 2-carbon atom of the end product of purine metabolism, uric acid (11). We have observed that folic acid antagonists inhibit the incorporation of radioactive formate into nucleic acid purines of mice (10), and that folic acid in large doses speeds up the leukemic processes in mouse leukemia Ak 4 (9). Aminopterin or A-methopterin (4-amino-N^{10}-methylpterolylglutamic acid) will consistently increase the life span of mice with leukemia, and this anti-leukemic activity may be reversed by folic acid (2, 3, 5, 7, 9) or by citrovorum factor (1). It has been suggested that formylfolic acid is a functional derivative of folic acid (4).

Shive *et al.* (6) observed that vitamin B_{12} at selected concentrations could replace both purines and thymidine in the nutrition of *Lactobacillus lactis* Dorner. Since it would appear from the above-mentioned evidence that folic acid antagonists block nucleic acid at the purine stage, it was considered of interest to determine if the anti-leukemic action of aminopterin or A-methopterin could be reversed by vitamin B_{12}.

EXPERIMENTAL

The anti-leukemic screening procedure employed in this investigation has already been described (8). All animals were maintained on the same diet of Purina chow. Leukemia-susceptible Akm mice were inoculated with Ak 4 leukemia, and treatment with aminopterin or A-methopterin alone, or in combination with B_{12}, was begun on the second post-inoculation day and continued on an alternate-day basis for a total of ten injections or until the animals died of leukemia. These compounds (the folic acid antagonists and B_{12}) were injected intraperitoneally from different syringes in immediate succession. Aminopterin was administered at the maximum tolerated dose of 0.23 mg/kg; A-methopterin, which is less toxic, was injected at the level of 2.0 or 3.0 mg/kg. In Experiment No. 2, Table 1, B_{12} concentrate was injected alone (0.06 mg/kg), to determine its effect on the pattern of deaths from leukemia. The levels of B_{12} concentrate[1] and crystalline B_{12}[2] used in other experiments are indicated in Table 1. The detailed results of all other experiments are also presented in Table 1. These data have been further summarized in Table 2.

DISCUSSION

The results of the eight separate experiments (each with internal control groups) provide a comparison of 90 folic acid antagonist-treated mice with 138 antagonist plus B_{12}-treated mice. The partial reversal of the anti-leukemic activity of the folic acid antagonists by B_{12} concentrate and crystalline B_{12} has been consistent, although not nearly so marked as is produced by folic acid.

A statistical analysis of the data presented on the partial reversal of A-methopterin by B_{12} has been carried out. The arithmetic mean of the life span of leukemic mice receiving the antagonist only (Exp. Nos. 3–7), when the two extreme observations (34 and 37 days) are eliminated, is 18.8 days. The mean life span of the mice receiving A-methopterin plus B_{12} in the same series of experiments is 16.2 days. A conventional "null" test for the significance of the difference between the two means shows that the discrepancy between them (2.6 days) is so great that it could occur as a result of chance about 1 time out of 100,000; thus, the difference between the two means is not fortuitous, and the facts are not consistent with the "null" hypothesis that the two are drawn from the same parent population.

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[1] B_{12} Squibb (a vitamin B_{12} concentrate derived from *Streptomyces griseus*).

[2] Crystalline B_{12} Merck (Cobione).

The possibility that the B_{12} concentrate used in these experiments contained folic acid or folic acid activity has been examined. Microbiological assays of the folic acid content of the B_{12} concentrate employed indicated almost no growth-promoting activity for *S. faecalis* in the absence of folic acid. The B_{12} concentrate was found to have about 0.13 per cent of the "folic acid activity" of commercial folic acid. Since 0.06 mg/kg of the concentrate partially reversed the anti-leukemic activity of the antagonists in mice, it would be necessary to assume that 0.00078 mg/kg of folic acid could produce the present results in order to explain these data on a "contamination" basis. The crystalline B_{12} results further confirm the postulate that B_{12} acts to partially reverse folic acid antagonists.

It would appear from these results and those reviewed in the introduction of this paper that B_{12} in some obscure fashion is helping to provide chemical moieties (nucleic acid precursors) which are deficient in the leukemic cells of mice treated with folic acid antagonists. Such an interpretation is compatible with Shive's observation that B_{12} is involved in the biosynthesis of purines (or derivatives) as well as thymine in certain bacteria.

TABLE 1

THE EFFECT OF VITAMIN B_{12} ON THE ANTI-LEUKEMIC ACTION OF FOLIC ACID ANTAGONISTS

Exp. no.	Treatment	Dosage (mg/kg)	Days from leukemic inoculation to death																													Average leukemic life (days)
			6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	32	33	34	37			
1	Controls	.....		2		4	4																									9.0
	Aminopterin	0.23								1	1		1	3	1		3															17.2
	Aminopterin +B_{12}	0.23+0.06								2	5				2		1															15.5
2	Controls	.....	1	1	3	3	2																									8.4
	B_{12}	0.06		2	4		4																									8.6
	Aminopterin	0.23							1			3	4		2																	15.7
	Aminopterin+B_{12}	0.23+0.06							1	4		1	3		1																	14.5
3	Controls	.....		2	1	5	2																									8.7
	A-methopterin	3.0										2	1		1	2		1		1				1			1					21.0
	A-methopterin+B_{12}	3.0+0.06							3		1		2			4																15.4
4	Controls	.....	3	1	1	4			1																							8.1
	A-methopterin	3.0												4			4	1					1									19.5
	A-methopterin+B_{12}	3.0+0.06					1	1		2		1		3			2															15.3
5	Controls	.....		3	3	4																										8.1
	A-methopterin	2.0											1	1		7															1	20.3
	A-methopterin+B_{12}	2.0+0.06							1				1			7																16.1
	A-methopterin+B_{12}	2.0+0.12							4	1			1		1	2								1								16.0
6	Controls	.....		1	5	3	1																									8.4
	A-methopterin	2.0								1			1			4		1	1	2												19.4
	A-methopterin+B_{12}	2.0+0.06								1		1	2	3		2		1														17.0
7	Controls	.....		8	1	1																										7.3
	A-methopterin	2.0									2		3	1	1	2	1															16.9
	A-methopterin+B_{12}	2.0+0.06								1	4	1		1	1																	14.9
8	Controls	.....		3	5	1	1																									8.0
	A-methopterin	2.0								1	1		1	1		4	1	2				5	1			2	1					22.1
	A-methopterin+B_{12}	2.0+0.06							1				3	2		4	1	2	1	1		3		1	1							20.4
	A-methopterin+B_{12}	2.0+0.3							2	1			3	1	4		2	2	2	2		1										18.6
	A-methopterin+B_{12}	2.0+0.6					1		1	1		2	1	2	2	1	2	1	2	3		1										18.5

NOTE: In Exp. Nos. 1, 2, 3, 4, 5, and 8, B_{12} concentrate was employed. In Exp. Nos. 6 and 7, crystalline B_{12} was injected.

TABLE 2

SUMMARY OF THE OBSERVATIONS ON REVERSAL OF THE ANTI-LEUKEMIC ACTION OF FOLIC ACID ANTAGONISTS

Exp. no.	R.C.I.*
1	0.78
2	0.84
3	0.54
4	0.63
5	0.66
	0.65
6	0.78
7	0.79
8	0.92
	0.84
	0.84
Av. (138 mice)	0.75

* Relative chemotherapeutic index—ratio of the per cent increase in life span of groups of mice treated with an antagonist plus B_{12} to the life span of the groups treated with antagonist alone.

SUMMARY

A consistent but small reduction in the efficacy of two folic acid antagonists in the treatment of Ak 4 leukemia in Akm mice was observed following the administration of vitamin B_{12}.

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The Effect of Regeneration on the Growth Potentialities *in Vitro* of Rat Liver at Different Ages

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It is known from the experimental work of numerous investigators (9, 32, 35, 40) on skin carcinogenesis that surgically-induced cell regeneration is able to cause an increase in tumor production when properly combined with the action of a specific carcinogen. As a general rule, the method used to demonstrate this effect consists of treatment of the skin by a suboptimal dose of a carcinogen, followed by excision or cauterization of a small area of the treated skin. The subsequent process of wound healing was found to have a definite enhancing effect on tumor formation (13, 19, 31, 40). Similar results have been obtained by the use of nonspecific physical (10, 34) or chemical (2, 6, 13, 35, 37, 39) irritants instead of the surgically-induced wound.

As a result of this work, the concept has been developed that carcinogenesis proceeds in a series of successive stages. Various ways of dividing the carcinogenic processes into stages have been proposed by several investigators (2, 3, 13, 23, 24, 36, 42). However, the primary hypothesis, common to all, first stated clearly by Rous (35), is the existence of a stage characterized by the formation of cells capable of forming tumors but not asserting themselves in this way unless stimulated to do so. Noncarcinogenic irritants or wound healing can act as stimuli during this stage. Thus, it appears probable that the mechanism of action of the irritants is somewhat similar to wound healing in the sense that both are phenomena involving cell regeneration.

In previous work the relationship between rat liver regeneration and carcinogenesis by *p*-dimethylaminoazobenzene was studied (16, 17). The experimental procedure consisted of the induction of vigorous regeneration by means of partial hepatectomy in livers which were in the process of evolution towards neoplasia after a limited exposure to the azo dye. The increased incidence of tumors subsequent to regeneration was considered to reflect the impact of regeneration upon the carcinogenic process.

The results of this work confirmed the concept that carcinogenesis is not a continuous single process but rather a series of successive cellular changes. It was shown that during the evolution of liver cells towards the neoplastic state and under certain conditions a particular cellular state may arise. This state was characterized by the fact that, although no sign of neoplasia was apparent, tumors developed rapidly when the cells were induced to regenerate. The question arose whether the age of the animals was one of the conditions influencing the manifestation of the enhancing effect of regeneration upon the carcinogenic process (17). Relative to this question, the findings of MacNider (20) on the influence of age on both the morphology and the biological properties of regenerating tissues would appear to have particular interest. He found that the regeneration process which follows cell injury varies according to the age of the animal. When epithelial tissues in young animals participate in a process of repair, the type of cell formed is normal and has no acquired resistance to subsequent injury. When a similar type of injury is produced in adult or senescent animals, there is shown by such animals either an inability to regenerate or a regeneration effected by an atypical type of cell, resembling, in certain aspects, embryonic epithelium which in addition has acquired a resistance to subsequent injury.

There exists a striking analogy between this type of regeneration following injury in old animals and the first phase of chemical carcinogenesis. In chemical carcinogenesis the immediate effect of the application of the carcinogen is injury to the tissue, followed by secondary regeneration. The regenerated epithelium shows an increased resistance to the toxic effects during further application of the carcinogen. It is this regenerating epithelium which forms the basis for the subsequent development of neoplasia. This sequence of events is apparent for both skin and liver carcinogenesis induced by either carcinogenic hydrocarbons or azo dyes,

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respectively (8, 29). Moreover, Miller and Miller (22) recently demonstrated that with the continuous administration of 4-dimethylaminoazobenzene to rats an initial increase of bound aminoazo dye in the liver is followed by a decrease with eventual disappearance in the arising tumors. This growing inability of the liver cells to bind azo dyes is very likely related to their increased resistance to the toxic effects of the azo dye.

Given these facts, one way by which more information could be obtained regarding the complicated relationship between regeneration and carcinogenesis would be the further study of the properties of regenerating tissues, taking into special consideration the factor of age. Accordingly, the tissue culture method was used in testing the growth capacity of liver tissues before and after regeneration was induced by partial hepatectomy in rats of varying ages.

MATERIALS AND METHODS

A total of 41 male rats was used for this experiment. Three experimental groups were formed: (*a*) 10 young animals, 4–8 weeks old; (*b*) 11 adult animals, 4–8 months old; and (*c*) 12 old animals, 18–24 months old. These three groups were used for a comparative study of normal and regenerating liver 72 hours after partial hepatectomy. A fourth group, containing 9 animals ranging from 9 to 19 months of age, was used for a comparative study of normal liver, regenerating liver at 72 hours, and regenerated liver at 45 days after partial hepatectomy.

The experimental procedure employed on each animal was as follows: A partial hepatectomy involving the median and the left lateral lobes, or 68 per cent of the liver, was performed under aseptic conditions. Part of the median lobe was immediately explanted in roller tubes, according to the technic of Gey (14, 15). Four tubes containing six fragments each were set up from each liver. The solid phase of the medium consisted of 10 per cent balanced salt solution (Gey), 10 per cent bovine embryo extract, 40 per cent human placental cord serum, and 40 per cent chicken plasma. The liquid phase consisted of 16 per cent balanced salt solution, 16 per cent bovine embryo extract, and 66 per cent human placental cord serum. In addition to this medium, two of the tubes received streptomycin and penicillin, 50 units each. There was no effect on the growth from these antibiotics.

In order to test the growth capacity of the liver itself rather than to observe the long-range effect on the tissue of the complex organic media used, the observation period was limited to 6 days, with individual observations every 24 hours and without renewal of medium. The growth capacity was estimated on an all-or-none basis, with the beginning of fibroblastic outgrowth, which was found always to precede epithelial outgrowth, the criterion. Therefore, livers showing fibroblastic outgrowth within 6 days were considered positive; those having no outgrowth, negative. In the cases where growth occurred, the latent period, or the interval between the explantation of the tissue and the time the first fibroblasts were observed, was taken into consideration.

In the first three experimental groups, 72 hours after the partial hepatectomy, the rats were killed, part of the regenerating right lateral lobe explanted, and its growth capacity tested as described above.

In the fourth experimental group, 72 hours after partial hepatectomy, an exploratory laparotomy was performed, and a small part of the right lateral lobe was excised and explanted. The rats were sacrificed 45 days later and observed as described above.

In order to investigate the pattern of *in vitro* growth of rat liver tissues and the possible effect of regeneration upon it, a number of cultures were carried for longer periods—up to 2 months, with renewal of the medium every 3 days.

RESULTS

Under the experimental conditions described and with the criteria used, it was found that the growth capacity of normal rat liver is an inverse proportional function of the age of the animal (Table 1). The percentage of livers showing outgrowth dropped from 100 per cent, in the case of young rats, to 45 per cent in the case of adults,

TABLE 1

LIVER	RATS	LIVERS explanted	LIVERS showing outgrowth	Percentage	Latent period in hours
NORMAL	Young	10	10	100%	58
	Adults	11	5	45%	82
	Old	12	1	8%	96
REGENERATING	Young	7	7	100%	31
	Adults	11	11	100%	33
	Old	8	8	100%	31

The growth capacity of rat liver is estimated on the basis of (*a*) the percentage of explanted livers which showed outgrowth *in vitro* during a test period of 6 days after explantation and (*b*) the latent period. The upper part of the table shows the findings on the normal livers from (*a*) young rats 4–8 weeks of age; (*b*) adult rats 4–8 months of age; (*c*) old rats 18–24 months of age. The lower part shows the findings on the same livers 72 hours after regeneration was induced by partial hepatectomy.

and to 8 per cent in the case of old animals. The variability of the latent period and its tendency to increase are also characteristic of the decline in growth capacity with advancing age.

Regeneration has a marked effect on this relationship between age and growth capacity. The percentage of regenerating livers which showed outgrowth 72 hours after partial hepatectomy remained at the figure 100 per cent throughout the age groups. The latent period, 31–33 hours, also showed a striking consistency in all age groups, and its difference from the values of normal liver was statistically significant.[1] Therefore, it appears that the growth capacity of regenerating liver is independent of the age of the animal.

It is known that at 72 hours after partial hepatectomy the mitotic activity of the liver *in vivo* is high. According to the work of Brues and co-workers (4), 0.63 per cent of the liver cells are in mitosis at 72 hours. In normal liver only 0.005 per cent of the liver cells are in mitosis. Consequently, the question arose whether the increased growth capacity of the regenerating tissue *in vitro* was related to the active mitotic process which this tissue was undergoing *in vivo* at the time of its explantation. To answer this question, partial hepatectomies were performed on a large number of rats ranging in age from 9 to 20 months, and their livers were explanted. From this group, nine rats were chosen because their normal livers did not grow *in vitro*. These rats constituted the fourth experimental group in which the growth capacity of regenerated liver was investigated 45 days after the partial hepatectomy and therefore long after the cessation of mitotic activity.

The results are shown in Table 2. It was found that at 45 days, as well as at 72 hours, all nine livers showed outgrowth. The latent period, however, in each individual case with the exception of one (No. 8) had increased. Thus, the average latent period of 80 hours was significantly different from the 34-hour figure characterizing regenerating liver 3 days after operation. These results indicate that the growth capacity *in vitro* of liver which has undergone regeneration does not depend on the mitotic activity *in vivo*. The increased latent period, however, showed that the growth capacity under these conditions has a definite tendency to decrease rapidly with time.

The study of the morphology and organization of the cultures showed that during the first days after explantation the patterns of growth of normal and regenerating tissues were identical (Fig. 1). In general, the cells grew out of the central fragment in the following sequence: macrophages, fibroblasts, and cells which appeared to be epithelial but which could not be identified with certainty at this early stage.

TABLE 2

RATS		NORMAL LIVER		REGENERATING LIVER at 72 hours		REGENERATED LIVER at 45 days	
NUMBER	AGE IN MONTHS	GROWTH	LATENT PERIOD IN HOURS	GROWTH	LATENT PERIOD IN HOURS	GROWTH	LATENT PERIOD IN HOURS
1	19	-	-	+	48	+	72
2	12	-	-	+	24	+	96
3	12	-	-	+	48	+	96
4	14	-	-	+	48	+	72
5	12	-	-	+	24	+	144
6	9	-	-	+	24	+	48
7	9	-	-	+	24	+	72
8	11	-	-	+	48	+	48
9	11	-	-	+	24	+	72
9	12	-	-	+	34	+	80

Individual findings on nine rats, concerning the growth capacity of their livers 72 hours and 45 days after the induction of regeneration. Average values are shown at the bottom of the table.

In the large majority of cases differences in the patterns of growth began to appear after 3 or 4 weeks of cultivation *in vitro*. At this time, in the case of normal adult liver (Fig. 2), the macrophages decreased in number, and the predominant cells were fibroblasts which formed a network. Isolated epithelial cells were also present and for the most part could now be identified as liver cells because of their large size and general morphology. The growth of epithelial cells as isolated units was the distinguishing feature of normal adult liver in these cultures. On the other hand, the cultures of regenerating liver from rats of all ages and of normal liver from young animals were characterized by a far greater outgrowth of liver cells and a definite tendency toward membrane formation (Fig. 3). This tendency illustrated the organization characteristic of regenerating and of normal young liver and resulted in the formation of large, loose epithelial sheets (Figs. 4, 5).

[1] Significance factor: $t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\epsilon_1^2 + \epsilon_2^2}}$.

$\bar{x}_1$ average of one group and ϵ_1 its standard error.

$\bar{x}_2$ average of second group and ϵ_2 its standard error.

For the difference in latent period between normal young liver and regenerating liver,

$$t = \frac{58 - 33}{\sqrt{5.65 + 3.82}} = 3.67, \mathrm{P} < 0.01 .$$

P probability of difference as great or greater than that obtained being due to chance.

DISCUSSION

The results on the decrease of the growth capacity *in vitro* of liver tissues with advancing age are in good agreement with the work of Emmart (11) on liver and of Carrel and other investigators on other tissues (7, 12, 18). It is also known that *in vivo* tissues display the same relationship between age and growth potential. The rate of wound healing was shown by Lecomte du Noüy to be an inverse proportional function of the age of the organism (27, 28).

Previous work (5) on liver has shown that the rate of liver cell restoration *in vivo* after partial hepatectomy declines with the advancing age of the animal. Marshak and Byron (21) in their studies of regenerating rat liver demonstrated that age delayed mitosis, i.e., the interval between hepatectomy and the time at which mitosis reached its peak increased with the age of the animal.

The decrease of the growth potential with age seems to be a universal phenomenon. In addition to the above examples of tissues of higher organisms, work on lower organisms supports this view (1, 30, 38, 43). Decline of growth and regeneration rates with increasing age in Crustacea has been recently described by Needham (26).

Our results show that regeneration has a marked effect on the relationship between age and growth potential. Under the conditions used in this experiment, regeneration resulted in complete restoration of the liver growth potential of old rats to the level of the young ones, as shown by the dramatic increase of the growth capacity from 8 to 100 per cent. Moreover, it was shown that the pattern of growth and organization of the colonies of regenerating liver was similar to the colony pattern of the normal resting liver of young rats. Therefore, it seems reasonable to assume that the qualitative difference in the patterns of organization of normal young liver and regenerating liver of all ages, on the one hand, and of normal adult and old liver, on the other, is a manifestation of the quantitative difference in their growth potential.

The increase of the growth potential brought about by regeneration outlasts the regenerative process itself. In old rats, the increased growth potential of the tissues formed after partial hepatectomy was still manifested 1 month after the completion of regeneration. However, the increased latent period at this time seems to indicate rapid aging of the regenerated tissue.

The effectiveness of cell multiplication and growth in interfering with aging processes was demonstrated by Sonneborn's work on the flatworm Stenostomum (41). If growth potential may be taken as an indication of age, an analogy may be drawn between this experiment and the work of Sonneborn in that regeneration of the liver of old rats seems to result in a temporary rejuvenation of the tissue.

In comparing this work with that of MacNider (20), some general conclusions may be drawn. Although MacNider used toxic compounds to induce regeneration and in this work partial hepatectomy was used, both approaches indicate that cell regeneration in young tissues does not alter their biological characteristics. However, in old tissues marked changes may appear as a result of regeneration.

According to MacNider, administration of uranium chloride to old dogs results in changes in hepatic epithelium that are correlated with the morphology and with the resistance to toxic factors. His description of an atypical, flattened syncytial liver epithelium resembling the embryonic type is certainly suggestive of a tissue of increased growth potential. In this paper evidence of a substantial increase in the growth potential of liver tissues following partial hepatectomy in old rats has been presented.

Although much further work is necessary to clarify the role of age in carcinogenesis, these findings suggest that the manifestation of the enhancing effect of regeneration upon the carcinogenic process could be expected to be greater with the increasing age of the animal.

SUMMARY

1. The growth potential of normal rat liver was tested by means of the tissue culture method. Under the experimental conditions used, the growth potential was found to be an inverse proportional function of the age of the animal.

2. The growth potential of regenerating rat liver was found to be independent of the age of the animal and of the same order of magnitude as that of the normal liver of young animals.

3. The restorative effect of regeneration on the growth potential of liver of old animals outlasted the regenerative process itself and was still apparent 1 month after the completion of regeneration.

4. Differences in the morphology and organization of the colonies of normal liver at different ages and of regenerating liver were described and their relationship to growth potential considered.

5. The significance of these results with respect to the general physiology of growth and carcinogenesis was discussed.

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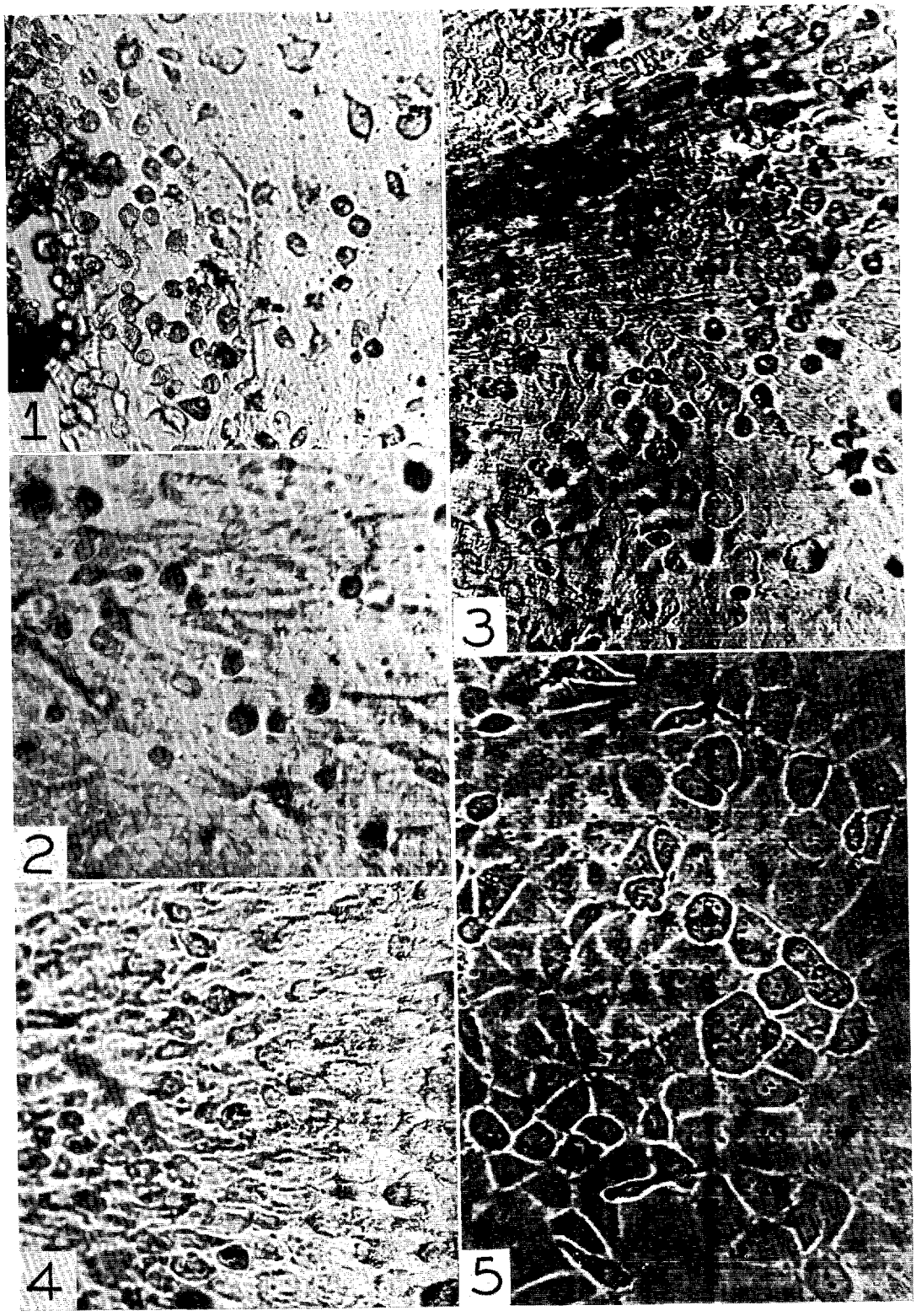

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FIG. 1.—Living culture of normal liver from a young rat 6 weeks of age. Four days *in vitro*. Outgrowth composed of fibroblasts, macrophages, and rounded epithelial cells. ×150.

FIG. 2.—Living culture of normal liver from an adult rat 8 months of age. Eighteen days *in vitro*. Outgrowth composed mostly of fibroblasts and separated epithelial cells. ×150.

FIG. 3.—Living culture of regenerating liver from the same rat as in Figure 2. The liver was explanted 72 hours after partial hepatectomy. Twenty days *in vitro*. Outgrowth composed mostly of numerous rounded epithelial cells and an epithelial membrane. ×150.

FIG. 4.—Living culture of regenerating liver from an adult rat 8 months of age. Thirty days *in vitro*. Outgrowth composed of extensive epithelial membrane. ×150.

FIG. 5.—Living culture of normal liver from a young rat 6 weeks of age. Twenty days *in vitro*. Outgrowth composed of extensive epithelial membrane with a few rounded cells lying on it. ×250.

Quantitative Evaluation of Growth Rates in Tumors before and after Radiation*

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The author is engaged in developing a classification of tumors according to their rates of growth, metabolic activities, and radiosensitivities. The need and feasibility of such a classification arose from the observations made in previous studies (9, 10, 12), in which it was demonstrated that the above characteristics may differ significantly even among tumors of almost identical morphological structure.

The purpose of the research reported herein is a quantitative evaluation of growth activity in tumors on a biological basis. As will be shown, the usual external physical measurements, made with a caliper, are not accurate criteria for judging the growth rates of tumors, particularly in relation to mitotic activity. For this investigation two tumors which had been previously studied extensively by the author were used. These were the mammary tumors of the Bar Harbor strains of mice, dba and C3H, both diagnosed as adenocarcinomas. For the sake of brevity, these tumors will be referred to as the dbrB and C3H tumors, respectively. A brief summary of the pertinent characteristics of each will follow.

The dbrB mammary tumor has a latent period of about 5–6 days, i.e., it reaches a size of about 6 mm. in diameter 5–6 days after implantation of a tumor graft. Within the following 8 days, the tumor increases rapidly in size, reaching a diameter of about 20–30 mm., and kills the animal within 3 weeks following implantation. The C3H mammary adenocarcinoma has a latent period of 14–18 days upon implantation of a tumor graft. The tumor increases in size slowly and kills the animal within about 3 months.

The rates of growth of these tumors were originally determined in the usual manner, i.e., by external measurement of two or three dimensions with a caliper. The question arose whether the difference in the increase in size of the above-mentioned tumors can be accounted for by a quantitative difference in mitotic activity or by other factors.

To throw light on this problem, a procedure which evaluates the growth activity of tumors on a more quantitative biological basis than external measurements alone is required. Such a procedure is particularly important for the evaluation of tumor therapy. It was decided to test the applicability of the method devised by Chalkley to the quantitative study of mitotic activity in tumors (4). This method permits a quantitative evaluation of spatial distribution of morphologic tissue components in an extended volume of tissue. It has been applied in studying the quantitative relationships of various tissue components by a number of investigators (2, 3, 5, 7, 14). However, to the knowledge of the author, it has not been employed for studies of the nature herein reported.

PART I

THE DETERMINATION OF VOLUME RATIOS OF RESTING TO MITOTIC CELLS IN THE C3H AND dbrB TUMORS

EXPERIMENTAL

Small fragments of young, actively growing dbrB and C3H mammary tumors, fixed in Zenker's solution and prepared in the usual manner, were used. Sections of about 4 μ in thickness were cut and stained with hematoxylin and eosin, and Feulgen. The latter has been helpful in identifying cells in active division.

Originally, due to the complexity of tumor tissue, several factors were chosen as criteria, such as a number of "hits" on resting cells, on cells in active division, on disintegrated cells, on hemorrhagic areas, and on empty spaces; but it was soon realized that such a variety of factors made the analysis too complex. The most reproducible ratios obtained were those of mitotic to intact

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resting tumor cells. It was decided to use these ratios as the criterion in the present study. The term "mitotic cell," as used in this paper, refers to those cells in division, from the earliest recognizable prophase to the separation of daughter cells. The term "resting cell" includes those cells which are intact and not in active division.

TABLE 1

VOLUME RATIOS OF MITOTIC TO RESTING CELLS OF dbrB TUMORS

Number of mouse	Age of tumor (days)	Mitotic index*
I	5	1:43.8
II	10	1:53.8
III	10	1:50.0
IV	13	1:53.8
V	15	1:46.7
Average of 5 tumors		1:49.6

* Ratio of dividing to resting cells in an extended volume of tissue.

Microscopic fields, showing intact portions of the tumors, were chosen at random for analysis. The number of mitotic cells hit during the counting of 700 hits on resting tumor cells within these intact portions formed the basis for the ratios. The choice of 700 hits for individual analyses of tumors is based on Chalkley's original investigations, in which he demonstrated that stabilizing ratios could be obtained from 500 hits, taking nuclei and cytoplasm as objects (4). To be even more precise, counts based on 700 hits were taken as a criterion for this investigation. The ratio for each tumor section so analyzed was obtained by dividing the 700 hits on resting cells by the number of hits on mitotic figures. The data obtained from five actively growing dbrB tumors are recorded in Table 1.

The reproducibility of the counts on one slide studied by several individuals and the narrow range of the results from five tumors of different ages justify the reporting of these observations.

RESULTS

Analysis of the data in Table 1 reveals that the volume ratios of mitotic to resting tumor cells range from 1:43.8 to 1:53.8, the average being 1:49.6. The age of the tumor, i.e., the time elapsing from implantation of the tumor fragments to the removal of the tumor for cytological analysis, varied from 5 to 15 days. This period is the time during which the tumor increases in size most rapidly. The average increase in tumor size in five mice within this period was $23 \times 15 \times 10$ mm. The relatively small variation of the volume ratios of mitotic figures to resting cells of these five tumors during their most active period of proliferation seems to indicate that mitotic activity alone does not entirely account for the rapid increase in dbrB tumor size. The presence of a considerable number of cystic empty spaces in the tumor sections (Fig. 1) offers a possible explanation for this sudden increase in tumor size, which is not due to mitotic activity. This point will be considered further in the discussion.

Table 2 records the relative volume ratios of mitotic to resting cells of five transplanted mammary adenocarcinomas of the C3H strain of mice. These ratios average 1 mitotic cell to 72.5 resting cells, ranging from 1:63.6 to 1:87.5.

Since the C3H tumor is the more slowly growing one, these tumors are considerably older than the dbrB tumors. The greatest increase in size occurs within 3 or 4 weeks following implantation, averaging about $9 \times 7 \times 6$ mm. From the data noted in Table 2, it appears that this tumor (C3H) reaches a rather even growth activity within 3 weeks following the latent period, as seen from the ratios of mitotic to resting cells.

TABLE 2

VOLUME RATIOS OF MITOTIC TO RESTING CELLS OF C3H TUMORS

Number of mouse	Age of tumor (days)	Mitotic index*
I	10	1:87.5
II	14	1:63.6
III	41	1:77.7
IV	41	1:63.6
V	57	1:70.0
Average of 5 tumors		1:72.5

* Ratio of dividing to resting cells in an extended volume of tissue.

PART II

THE DETERMINATION OF VOLUME RATIOS OF RESTING TO MITOTIC CELLS IN dbrB TUMORS FOLLOWING IRRADIATION

In studying the effects of irradiation, there is a great need for an index evaluating the efficacy of radiation on living tissues, particularly where therapy of malignant tumors is concerned. It has been shown that not all cells can be destroyed outright, unless tremendous radiation doses are applied. This information was obtained from experiments carried out in tissue culture *in vitro* (6, 8, 12, 13, 15) and *in vivo* (10). However, large doses cannot be employed *in vivo* in one exposure due to the damaging effect on normal tissues, primarily on the skin. Consequently, in radiation therapy the total dose required for tumor destruction has to be divided, i.e., fractionated into

smaller ones and applied within certain intervals of time.

In applying fractionated treatments, one faces the problem of permitting the uninjured tumor cells to continue to grow and the partially injured tumor cells to recover before a subsequent radiation dose is given. It is of importance, therefore, to ascertain the extent of damage done to the tissue and the extent of its recovery following each radiation dose, in order to adjust the over-all time within which the course of treatment should be carried out and thus achieve the desired results. A quantitative procedure for evaluating such a situation would be of great value. The satisfactory results obtained from the evaluation of growth activity of nonirradiated tumors by the use of Chalkley's method prompted the application of this method to the study of irradiated tumors. The dbrB tumor was selected for this experiment because of its more uniform rate of growth; for example, its latent period is usually about 5–6 days.

Actively growing dbrB tumors, grown in dba mice, were exposed, *in situ*, to various doses of x-radiation (the physical factors: 200 kv; 20 ma; 0.5 mm. Cu + 1.0 mm. Al filtration and HVL = 1.1 mm. Cu. The tumors were irradiated at a distance of 12.5 cm. from the x-ray source, and the average intensity was 602 roentgens/min). The irradiated tumors were excised within various intervals of time following irradiation, and portions from several areas were immediately fixed in Zenker's solution. With the routine procedure, paraffin sections 4 μ in thickness were stained with hematoxylin and eosin, and Feulgen. Only intact areas of the tumor section in the microscopic field were chosen for analysis. This was done for two reasons: First, it was deemed of greater significance to know the activity of the intact portions of tumor tissue remaining after irradiation than to know how much had been destroyed; such a criterion may serve as a guide in planning further treatments with additional doses of irradiation. Second, this was necessary in order to be able to compare the results with those obtained from the nonirradiated, control tumors for which the same criterion was used.

In the irradiated tumors, as in the controls, the number of mitotic cells hit during the counting of 700 hits on intact resting cells within the same microscopic fields determined the ratios. No attempt is made here to describe in detail the histologic changes occurring in the tumors following irradiation, because such changes have been described elsewhere (10).

In Table 3 are recorded the results obtained from five dbrB tumors irradiated with different doses of x-rays and removed for analysis following various intervals of time. Analysis of the data revealed the following: A ratio of 1 mitotic cell to 41.0 resting tumor cells was found in intact portions of an 11-day-old dbrB tumor 24 hours following exposure to 5,000 r. This ratio lies close to the range noted in the control nonirradiated dbrB tumors. Whether these cells escaped being hit by x-rays or recovered following slight injury, or whether they might have shown some effect later on, known as a "delayed effect" produced by radiation, cannot be determined at the present time.

A ratio of 1 mitotic figure to 87.5 resting cells was found in a dbrB tumor 13 days following irradiation with 5,000 r. This ratio indicates a decrease in mitotic activity, as compared to the average ratio of normal control tumors, which was 1:49.6. A delayed effect of irradiation may be indicated in this case.

TABLE 3

VOLUME RATIOS OF MITOTIC TO RESTING CELLS OF dbrB TUMORS FOLLOWING IRRADIATION

Number of mouse	Dose (roentgens)	Post-radiation time	Mitotic index*
I	5,000	24 hours	1:41.0
II	5,000	13 days	1:87.5
III	10,000	26 days	1:100
IV	12,000	50 hours	1:87.5
V	16,000	22 hours	1:350

* Ratio of dividing to resting cells in an extended volume of tissue.

As expected, with the increase of the x-ray dose a decrease in mitotic activity occurred. A ratio of 1:100 was calculated from a tumor 26 days following exposure to 10,000 r, indicating a decrease in active proliferation of the intact portions of the tumor as compared to the average mitotic activity of control, nonirradiated tumors. A ratio of 1 mitotic to 87.5 resting intact tumor cells was found in a dbrB tumor 50 hours following exposure to 12,000 r, and a ratio of 1 mitotic to 350 resting tumor cells was calculated from a tumor 22 hours following exposure to 16,000 r.

As anticipated, there were variable amounts of disintegrated portions in the irradiated tumors, depending upon the dosage applied and the length of time following irradiation. However, this was not taken into consideration, because the main interest lies, not in the amount of tumor tissue destroyed, but in the proliferative potentialities of the tumor after irradiation.

The method was further applied to throw light on the problem of "indirect effects"[1] of radiation

[1] "Indirect effect" as used here refers to the effect resulting from toxic substances produced by irradiation and does not

using mitotic activity as a criterion. The theory advanced by some investigators concerning the effects of radiation on living matter is that a tumor situated in a region remote from the one which is being irradiated might be affected indirectly. For example, Ahlstrom *et al.*, working with phosphorus 32, showed changes in the nuclear DNA in a tumor opposite the one which was exposed to radiation (1). Such an "indirect effect" of radiation is explained by the presence of toxic substances circulating in the animal organism which were produced by irradiation.

The problem of "indirect effects" of radiation will not be discussed here. However, if such effects actually influence tumor growth, they should be reflected in the mitotic activity. Since detailed data bearing on these "indirect effects" will be presented elsewhere, only one example typical of these findings will be mentioned here.

A number of dba mice received grafts on both sides, between the groin and subaxillary regions, of dbrB tumor particles of similar size. The tumors on both sides developed equally well in all mice bearing implants, and the tumors situated on the right sides of the mice were exposed to lethal doses of x-radiation, while the rest of the animal organism was protected by the device described in a previous report (10). The example referred to above is as follows: A tumor on the right side received a total dose of 20,000 r, applied in four exposures of 5,000 r each, with a 24-hour interval between each exposure. Six days following the last treatment the tumor had regressed, and only some scar tissue remained, while the untreated tumor on the left side continued to increase in size (Fig. 2). The latter was excised and fixed; sections 4 μ in thickness were stained for cytological study. A ratio of 1 mitotic figure to 41.1 resting tumor cells was found in these sections. This volume ratio of mitotic to resting cells falls close to the range of those ratios found in normal, untreated dbrB tumors.

Numerous observations have been made in this laboratory of continued growth of untreated tumors, both adjacent to and remote from irradiated tumors which regressed following radiation. Examples of such observations were previously reported by the author (10, 11). The present finding, based on mitotic activity, is in accord with these observations. This suggests that the supposedly "indirect effect" of irradiation, presumably produced by toxic substances circulating in the organism was not, in this case, sufficient to destroy tumor cells or even to affect the rate of growth of a tumor autogenous to the strain of the mouse. The discrepancy between these observations and those reported by Ahlstrom *et al.* may be due to a different experimental procedure in irradiating the animals.

refer to the other definition involving interference with the vascular and connective tissue system of the tumor.

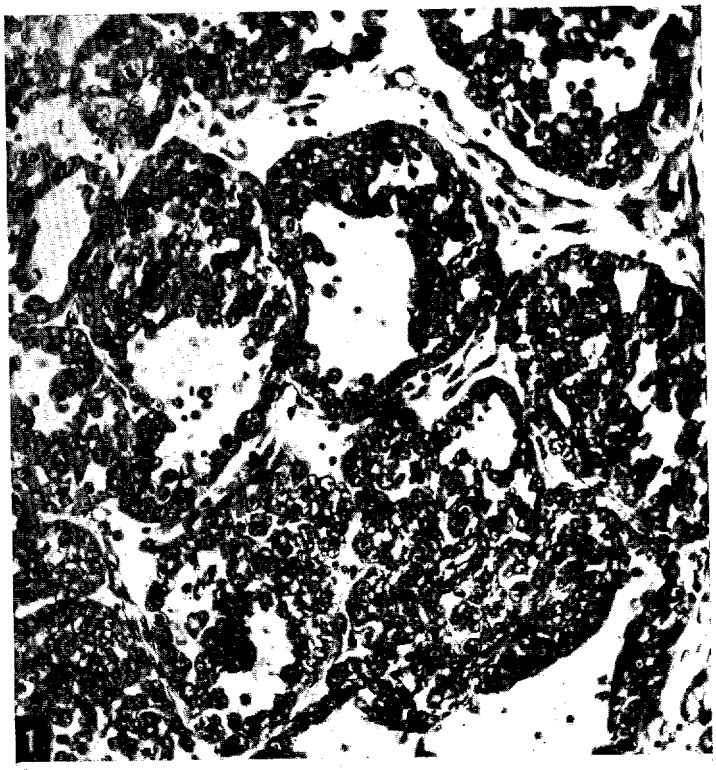
SUMMARY AND CONCLUSIONS

Attempts were made to evaluate the rates of growth of tumors before and after radiation on a quantitative biological basis. Chalkley's method, which permits a quantitative evaluation of spatial distribution of morphologic tissue components in an extended volume of tissue, was used for this study.

Two mammary tumors of the Bar Harbor strains of mice, dba and C3H, both diagnosed as adenocarcinomas and referred to in the text as the dbrB and C3H tumors, respectively, were employed as test objects. Analysis of five dbrB tumors yielded an average ratio of mitotic to resting cells of 1:49.6 (range: 1:43.8–1:53.8), while analysis of five mammary tumors of the C3H strain yielded an average ratio of mitotic to resting cells of 1:72.5 (range: 1:63.6–1:87.5). It is indicated that the relatively greater increase in size of the dbrB mammary tumor, as compared to the C3H tumor, is due not only to the greater mitotic activity of this tumor but also to its inherent secretory tendency, which produces engorgement of the glandular lumina. Consequently, it is thought advisable to take such a biological characteristic into consideration in evaluating the increase in tumor size as measured externally.

Volume ratios of mitotic to intact resting cells of five dbrB tumors, exposed to various doses of x-radiation and removed following various periods of time, are presented. Microscopic fields of stained sections of intact portions of the tumor, chosen at random, were used for analysis. A decrease in mitotic activity was noted which depended upon the dose of irradiation applied and the lapse of time between exposure and removal of the tumors. For example, 1 mitotic figure to 41.0 resting cells was found in the intact portions of a dbrB tumor 24 hours following a dose of 5,000 r; a ratio of 1:87.5 was found in another dbrB tumor which received the same dose of radiation but which was removed 13 days following exposure. The ratio of 1:41.0 is very close to the range of mitotic indices found in untreated dbrB tumors, while the ratio of 1:87.5 indicates a decrease in mitotic activity as compared to the normal. A delayed effect is indicated.

The effectiveness of a large dose of radiation is illustrated by the ratio of 1 mitotic figure to 350



1

2

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FIG. 1.—A section of dbrB tumor. Note the dilatation of the lumina of the acinic structures, some of which still contain mucoid material, and the empty spaces. ×260.

FIG. 2.—A mouse which received grafts in both subaxillary regions of tumor particles. The tumor on the right side regressed following x-radiation. Note epilation, regrowth of gray hair and some scarring on this site. Note the continuous growth of the untreated tumor seated in the left subaxillary region.

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resting cells found in a dbrB tumor which was removed and fixed 22 hours following irradiation with 16,000 r.

The results obtained indicate the possibility of applying Chalkley's method to the quantitative evaluation of growth rates of tumors, taking the mitotic index as a criterion. This method also proved helpful in evaluating the effectiveness of a given dose of radiation on a quantitative basis.

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Alkaline Phosphatase Substrate Specificities in Cultured Normal and Malignant Cells of Mouse, Rat, and Fowl*

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INTRODUCTION

It has been reported (1) that adenosine triphosphate or muscle adenylic acid gave greater blackening than yeast adenylic acid or sodium glycerophosphate when used as substrates in the Gomori test (4) for alkaline phosphatase on tissue cultures of embryonic mouse skin and mouse sarcomas T241 and Ma387. This finding has now been followed by more detailed studies on a variety of tumors and normal tissues from several species. It was our intention to determine whether or not malignant cells showed any consistent deviation from normal cells in the relative substrate specificity of their alkaline phosphatase. For this purpose, results with muscle adenylic acid were compared to those obtained with yeast adenylic acid and sodium glycerophosphate.

Muscle adenylic acid is preferentially dephosphorylated by the enzyme 5-nucleotidase, described by Reis (11, 12). Gulland and Jackson (6), as well as Reis, observed that extracts rich in 5-nucleotidase activity rapidly attacked muscle adenylic acid or inosinic acid but split such materials as yeast adenylic acid or glycerophosphate much more slowly. The purine riboside-5-phosphate grouping found in muscle adenylic acid is the specific substrate of the group II alkaline phosphatase enzymes of Newman and collaborators (9). It has been observed by Gomori (5) that alkaline phosphatase tests with muscle adenylic acid give different results from those with glycerophosphate in paraffin sections of certain organs. Hence, the studies reported below may be regarded as comparisons of the relative activities of nonspecific phosphomonoesterase and 5-nucleotidase.

The exactness of localization of alkaline phosphatase in the histochemical test has recently been questioned (3, 7), with evidence brought forward for the diffusion of the enzyme and its adsorption on nuclear structures, resulting in a possibly false picture of its distribution. With this in mind, one may interpret the results reported here in two ways: The intensity of staining on nuclear structures may be regarded either as bearing some relation to enzymatic activities on those structures in life or as reflecting activity of diffusible enzyme originating elsewhere in the tissue and adsorbed on the nuclear structures after death. That is, the interpretation is related to the tissue culture as a whole, and with some doubt it may apply to localized structures as well.

It was found that the various tissues, when cultured and fixed, showed different predilections for the substrates employed. Most notable was the considerably greater splitting of muscle adenylic acid than of the other substrates by Sarcoma 180 cells. However, there was no correlation between malignancy and the differential ability to attack any given substrate. Results of tests for phosphatase activity in mitotic chromosomes were paralleled by results in resting nuclei, in which the nucleolar complex of plasmosome and nucleolus-associated heterochromatin was specifically under consideration.

MATERIALS AND METHODS

Cultures were made of tissues from three species. The mouse tissues included sarcoma T241, lung tumor Ma387, Crocker sarcoma 180, Ridgway osteogenic sarcoma, and Carcinoma 1025, as well as four embryonic tissues: heart, skeletal muscle, liver, and brain. The rat tissues planted were sarcoma R39 and embryonic skin. The chicken tissues included Rous sarcoma cells taken from cultures on the chorioallantoic membrane, and embryonic skeletal muscle, brain, and intestine.[1]

Small fragments of these tissues about 1 or 2 mm. in diameter were planted from the animal on coverslip inserts in flattened roller tubes. Each

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roller tube held two coverslip inserts, and there were two or three explants on each coverslip, usually of different tissues. The explants were covered with a thin chicken plasma clot, and clotted plasma was also used to hold the coverslips to the roller tube wall. The incubation medium, 1 ml. per roller tube, was composed of 4 parts Gey's salt solution, 2 parts chick embryo extract, 1 part human placental serum, and 3 parts horse serum, with initial pH at 7.4–7.8. This medium contained 25 units each of penicillin and streptomycin per milliliter. The cultures were incubated at 37° C. for 1 or 2 days (i.e., until a sufficient growth had been obtained) in a drum rotating 10 times per hour.

After being rinsed in physiological saline, cultures were fixed in a solution suggested by Danielli (2). The fixative was composed of 70 parts ethyl alcohol, 20 parts pyridine, 5 parts 37 per cent formaldehyde, and 5 parts water. The fixative was made up fresh and chilled to −20° C. before use. The cultures were held in the fixative at this temperature overnight or for several days before the Gomori phosphatase test was carried out. Before the test, the explants were removed from the coverslips with fine forceps, with the zones of growth being left.

For the test, the coverslip cultures were incubated in Columbia staining dishes holding 10 ml. of distilled water containing sodium barbital, 2.1 mM/l; calcium chloride, 15 mM/l; magnesium chloride, 20 mM/l; and the substrate in concentrations ranging in fivefold steps from 0.0003 to 5.0 mM/l. Control cultures were incubated in this medium without an organic phosphate substrate. The pH of the medium was adjusted to 9 where necessary. Incubation was for 19 hours at 37° C. Thereafter, the coverslips were passed through 4 changes of 1 per cent calcium chloride and were then exposed to 2 per cent cobalt chloride for 5 minutes, a running distilled water rinse for 30 seconds, a 1 per cent dilution of ammonium sulfide by volume for 3 minutes, and a second running distilled water rinse for ½ minute. After being dehydrated in alcohol and cleared in xylene, the preparations were mounted in a toluene-soluble resin.

An attempt was made to estimate the extent of blackening by means of a microphotometer. This apparatus was patterned after that described by Pollister and Ris (10) and made use of a Photovolt photometer model 512 with a photomultiplier tube. Illumination of Köhler type was furnished by a tungsten ribbon filament lamp and was filtered through a glass water cell and a Corning blue daylight glass. The microscope condenser diaphragm was opened to admit only axial illumination through a circle 3 mm. in diameter. Readings were made with the slide under oil immersion. A diaphragm beneath the photocell opening admitted only that light passing through a circle of approximately 20 square μ area in the object plane.

In twenty nonmitotic cells taken at random in a given culture, readings were made of the light passing through a cylindrical portion of the cell, including a nucleolus and associated heterochromatin. The nuclear membranes and a thin sheet of cytoplasm above and another below the nucleus were also in the path of light, it must be remembered. With each such reading an additional reading through an adjacent portion of the clot free of cells was taken. The data in the tables are ratios of the galvanometer deflection found with light passing through the nucleus to that found in the clot reading.

Mean and standard error were determined for the twenty observations through resting nuclei per culture. In addition, readings in irregular numbers were taken through mitotic figures at stages of great chromosome condensation, namely, metaphase, anaphase, and early telophase.

Microphotometry was employed with reservations, which are mentioned in the discussion. Serving somewhat as a shorthand substitute for an extensive photographic record, the photometric data are to be regarded only as indications, with little quantitative significance, of the extent of blackening with the cobalt sulfide precipitate. This, in turn, may reflect only crudely the actual enzyme activity. Significant differences may well be those restricted to extensive blackening on the one hand and light browning or none on the other.

RESULTS

Results of some 5,000 observations with resting nuclei are summarized in Tables 1–4. Mean ratios of light transmission through a cylindrical portion of cell including some of the nucleus containing a nucleolus are given for various concentrations of the three substrates, together with standard errors. Results with the lowest concentrations in many sets are omitted from the tables to save space, because they agree with control values.

The mouse tissues could be grouped into roughly three classes with respect to their blackening with the substrates. Crocker mouse sarcoma 180 was outstanding in the far greater ease with which phosphatase activity could be demonstrated in its fixed cells with muscle adenylic acid than with yeast adenylic acid or glycerophosphate. Somewhat less pronounced in this same respect were

TABLE 1

MOUSE NORMAL TISSUES; RATIOS OF TRANSMISSION THROUGH NUCLEUS (NUCLEOLUS) TO THAT THROUGH ADJACENT CLOT

	Muscle Adenylic acid		Yeast Adenylic acid		Glycero-phosphate		No substrate	
Conc.	Mean	St. err.	Mean	St. err.	Mean	St. err.	Mean	St. err.
MOUSE EMBRYO SKIN:							0.92	±0.008
5.0 mM/l	0.28	±0.015	0.48	±0.020	0.63	±0.028		
1.0	0.50	0.029	0.70	0.027	0.77	0.018		
0.2	0.44	0.019	0.82	0.014	0.92	0.005		
0.04	0.46	0.041	0.92	0.007	0.92	0.009		
0.008	0.94	0.007	0.96	0.005	0.89	0.010		
MOUSE EMBRYO BRAIN:							0.88	0.017
0.04	0.39	0.030	0.47	0.045	0.88	0.013		
0.008	0.88	0.017	0.82	0.011	0.95	0.007		
MOUSE EMBRYO HEART:							0.89	0.016
5.0			0.48	0.020	0.49	0.024		
1.0	0.41	0.019	0.69	0.022	0.53	0.028		
0.2	0.43	0.023	0.62	0.034	0.44	0.031		
0.04	0.55	0.026						
MOUSE EMBRYO LIVER:							0.85	0.009
5.0	0.51	0.030	0.49	0.021	0.47	0.027		
1.0	0.45	0.028	0.66	0.040	0.62	0.031		
0.2	0.61	0.020	0.90	0.009	0.74	0.021		
0.04	0.71	0.026	0.84	0.024	0.92	0.011		
0.008	0.88	0.011	0.86	0.013	0.80	0.023		

TABLE 2

MOUSE TUMOR TISSUES; RATIOS OF TRANSMISSION THROUGH NUCLEUS (NUCLEOLUS) TO THAT THROUGH ADJACENT CLOT

	Muscle Adenylic acid		Yeast Adenylic acid		Glycero-phosphate		No substrate	
Conc.	Mean	St. err.	Mean	St. err.	Mean	St. err.	Mean	St. err.
CROCKER SARCOMA 180:							0.73	±0.013
5.0 mM/l			0.57	±0.020	0.70	±0.016		
1.0	0.17	±0.006	0.80	0.009	0.77	0.011		
0.2	0.21	0.009	0.84	0.019	0.84	0.032		
0.04	0.21	0.010						
SARCOMA T241:							0.88	0.015
5.0	0.15	0.011	0.31	0.017	0.40	0.025		
1.0	0.16	0.012	0.60	0.023	0.58	0.025		
0.2	0.18	0.015	0.65	0.018	0.75	0.011		
0.04	0.24	0.018	0.70	0.011	0.78	0.016		
0.008	0.73	0.019	0.73	0.014	0.78	0.015		
LUNG TUMOR MA387:							0.88	0.011
5.0	0.23	0.011			0.50	0.022		
1.0	0.30	0.018	0.65	0.019	0.72	0.016		
0.2	0.39	0.012	0.81	0.014	0.89	0.009		
0.04	0.61	0.036	0.81	0.017				
CARCINOMA 1025:							0.90	0.011
5.0	0.37	0.028	0.49	0.024	0.56	0.030		
1.0	0.48	0.025	0.59	0.020	0.72	0.021		
0.2	0.70	0.017	0.63	0.025				
0.04	0.76	0.024	0.93	0.008				
RIDGWAY OSTEOGENIC SARCOMA:							0.86	0.015
5.0	0.37	0.026	0.60	0.025	0.46	0.025		
0.2	0.57	0.023	0.50	0.027	0.54	0.022		
0.04	0.78	0.029	0.67	0.022	0.89	0.012		
0.0016	0.83	0.013	0.91	0.007	0.91	0.008		

sarcoma T241, lung tumor Ma387, Carcinoma 1025, embryo skin, and embryo brain. All three substrates were split with approximately equal ease in the third group, which included mouse embryo heart, liver, and skeletal muscle (for which no photometric data were collected), as well as Ridgway osteogenic sarcoma.

Fixed cultures of rat sarcoma R39 and rat embryo skin each split the two adenylic acids to about the same extent and glycerophosphate nearly so.

Fixed cultures of chick embryo brain, intestine, and skeletal muscle showed slightly but irregularly greater blackening with the adenylic acids than with glycerophosphate. Cultures of Rous sarcoma cells exhibited only weak alkaline phosphatase activity and a slight predilection, if any, for yeast adenylic acid among the three substrates.

The above results with phosphatase reactions in the nucleolus and associated chromatin in resting nuclei were paralleled in great measure by results with mitotic chromosomes. Photometric determinations were made on sets of cultures of mouse embryo skin, heart, and brain, and of Sarcoma 180, Carcinoma 1025, and chick skeletal muscle. In the results given in Table 5, each entry is the mean of determinations on 3–15 different mitotic figures.

The parallelism of results between nucleoli with associated heterochromatin in resting nuclei, on the one hand, and mitotic chromosomes, on the other, seen in a comparison of Table 5 with the previous tables, appeared to extend to all tissues planted, according to visual observations.

Most striking were results with Sarcoma 180 chromosomes. As with resting nuclei, Sarcoma 180 chromosomes were greatly blackened by cobalt sulfide after incubation with muscle adenylic acid but only weakly stained after incubation with the other substrates. Figures 1–3 are photomicrographs made under similar conditions of fixed cells

TABLE 3

RAT TISSUES; RATIOS OF TRANSMISSION THROUGH NUCLEUS (NUCLEOLUS) TO THAT THROUGH ADJACENT CLOT

	Muscle Adenylic acid		Yeast Adenylic acid		Glycerophosphate		No substrate	
Conc.	Mean	St. err.	Mean	St. err.	Mean	St. err.	Mean	St. err.
RAT EMBRYO SKIN:							0.92	±0.014
5.0 mM/l	0.57	±0.024			0.75	±0.022		
1.0	0.51	0.023	0.73	±0.026	0.59	0.023		
0.2	0.63	0.025	0.53	0.026	0.93	0.007		
0.04	0.93	0.006	0.91	0.009	1.00	0.002		
RAT SARCOMA R39:							0.94	0.007
5.0	0.26	0.028	0.26	0.028	0.47	0.040		
1.0	0.35	0.041	0.26	0.032	0.37	0.042		
0.2	0.53	0.011	0.38	0.042	0.56	0.042		
0.04	0.57	0.032	0.62	0.055	0.99	0.002		
0.008	0.91	0.012	0.92	0.008				

TABLE 4

CHICK TISSUES; RATIOS OF TRANSMISSION THROUGH NUCLEUS (NUCLEOLUS) TO THAT THROUGH ADJACENT CLOT

	Muscle Adenylic acid		Yeast Adenylic acid		Glycerophosphate		No substrate	
Conc.	Mean	St. err.	Mean	St. err.	Mean	St. err.	Mean	St. err.
CHICK EMBRYO BRAIN:							0.86	±0.012
5.0 mM/l	0.26	±0.025	0.54	±0.035	0.41	±0.028		
1.0	0.32	0.023	0.65	0.030	0.56	0.022		
0.2	0.54	0.039	0.52	0.041	0.65	0.008		
0.04	0.65	0.029	0.84	0.017	0.92	0.009		
0.008	0.82	0.012	0.82	0.012				
CHICK EMBRYO INTESTINE:							0.95	0.007
5.0	0.40	0.032	0.42	0.020	0.46	0.021		
1.0	0.44	0.013	0.51	0.026	0.63	0.016		
0.2	0.61	0.025	0.53	0.008	0.87	0.008		
0.04	0.94	0.007	0.93	0.005	0.91	0.005		
CHICK EMBRYO SKELETAL MUSCLE:							0.93	0.007
5.0			0.56	0.028	0.71	0.029		
1.0	0.63	0.018	0.82	0.017	0.81	0.021		
0.2	0.92	0.003	0.71	0.017	0.95	0.009		
ROUS SARCOMA FROM CHICK EMBRYOS:							0.94	0.012
5.0	0.65	0.020	0.46	0.037	0.51	0.026		
1.0	0.71	0.026	0.52	0.019	0.95	0.006		
0.2	0.96	0.008	0.97	0.004	0.90	0.012		

of Sarcoma 180 cultures incubated with the same concentration of the three substrates. The processing of the three photographs was identical.

A sharp contrast is furnished by the photomicrographs of embryonic mouse heart cells in Figures 4–6. These cultures were on the same coverslips as were the Sarcoma 180 cultures of Figures 1–3, yet the heart cultures were nearly equivalent in staining after all substrates.

Figures 7–18 illustrate the effect of change of substrate upon some of the other tissues. Each set of three figures from each tissue was prepared by similar photographic processes. The differences in density of background within each set are probably related to differences in clot thickness and staining, some of which may depend on differences in quantity of diffusible phosphatase in the clot derived from the cells.

TABLE 5

CHROMOSOMES; RATIOS OF TRANSMISSION THROUGH CONDENSED MITOTIC FIGURES TO THAT THROUGH ADJACENT CLOT

Conc.	Muscle Adenylic acid Mean	Yeast Adenylic acid Mean	Glycerophosphate Mean	No substrate Mean
MOUSE EMBRYO SKIN:				0.84
5.0 mM/l		0.39	0.52	
1.0	0.41	0.63	0.54	
0.2	0.43	0.57	0.74	
MOUSE EMBRYO HEART:				0.88
5.0		0.29	0.40	
1.0	0.35	0.58	0.36	
0.2	0.35	0.28	0.46	
MOUSE EMBRYO BRAIN:				0.77
0.04	0.24	0.25	0.71	
0.008	0.59	0.72	0.69	
CHICK EMBRYO SKELETAL MUSCLE:				
5.0		0.60	0.65	
1.0	0.73		0.77	
0.2		0.68	0.80	
MOUSE SARCOMA 180:				0.80
5.0		0.61	0.80	
1.0	0.14		0.71	
0.2	0.16	0.84	0.77	
MOUSE CARCINOMA 1025:				0.87
5.0	0.21	0.29	0.43	
1.0	0.31	0.53	0.47	
0.2	0.54	0.50		
0.04	0.61	0.89		

Experiments of both groups I and II of Newman and collaborators (9) on inhibitors indicated the presence of enzymes in tissue cultures of Sarcoma 180 and mouse embryo skin. The splitting of muscle adenylic acid was hindered less than was that of yeast adenylic acid by 10 mM/l sodium cyanide in the incubation medium. There was less difference in this respect in the presence of 250 mM/l glycine. There were equally great inhibitions with both adenylic acids after treatment of the fixed cultures with 5 per cent trichloroacetic acid at room temperature or with water at 80° C. for 10 minutes.

DISCUSSION

Increased objections have arisen of late to the use of the histochemical test for alkaline phosphatase activity (3, 7) because of the possibility that the enzyme or an intermediate product in the reaction is not securely localized. If it were to be accepted that much of the staining of nuclei in this histochemical procedure is an artifact caused by *post mortem* adsorption of diffusible enzyme on nuclear structures (3), then interpretation of our results would be considerably restricted. The data on nuclear blackening would be meaningless as far as nuclei are concerned and would serve to indicate only crudely the relative amounts of the diffusible enzymes originally present in the cultures, subject to possible differences in adsorptive capacity of the nuclei of the several tissues with respect to any one enzyme.

However, it is noteworthy in our material that the various tissues retained their characteristic activity patterns despite the fact that cultures of several different tissues were customarily planted on each coverslip within a few mm. of one another. Indeed, the substrate specificities supplemented purely morphological means of detecting cells from one culture that had wandered into the growth zone of another (Fig. 19). A fixation artifact involving movement of enzyme within the individual nucleus was evident in cells on the coverslip beneath the original explant, which was removed after fixation. In these nuclei, there was a movement of phosphatase-positive material in the presumed direction of movement of the fixative toward the center of the explant. The enzyme movement was apparently halted by the nuclear membrane (Fig. 20). This artifact was not evident in cells of the growth zone, where our interest lay. It would appear that the movement of enzyme in our material could not have been extreme under the conditions employed.

The microphotometric apparatus served as a convenient means of assessing the approximate density of the stain. The results are to be regarded at best as only coarse indications of relative enzyme activity. In many cases the several highest concentrations of substrate gave equivalent small deflections of the galvanometer, much as several of the lowest concentrations caused equivalent great deflections; with these lowest concentrations, as with controls, light loss apparently occurred largely because of structural light scattering and because of the staining of preformed phosphate. Frequently, only a few intermediate concentrations caused an intermediate deflection of the

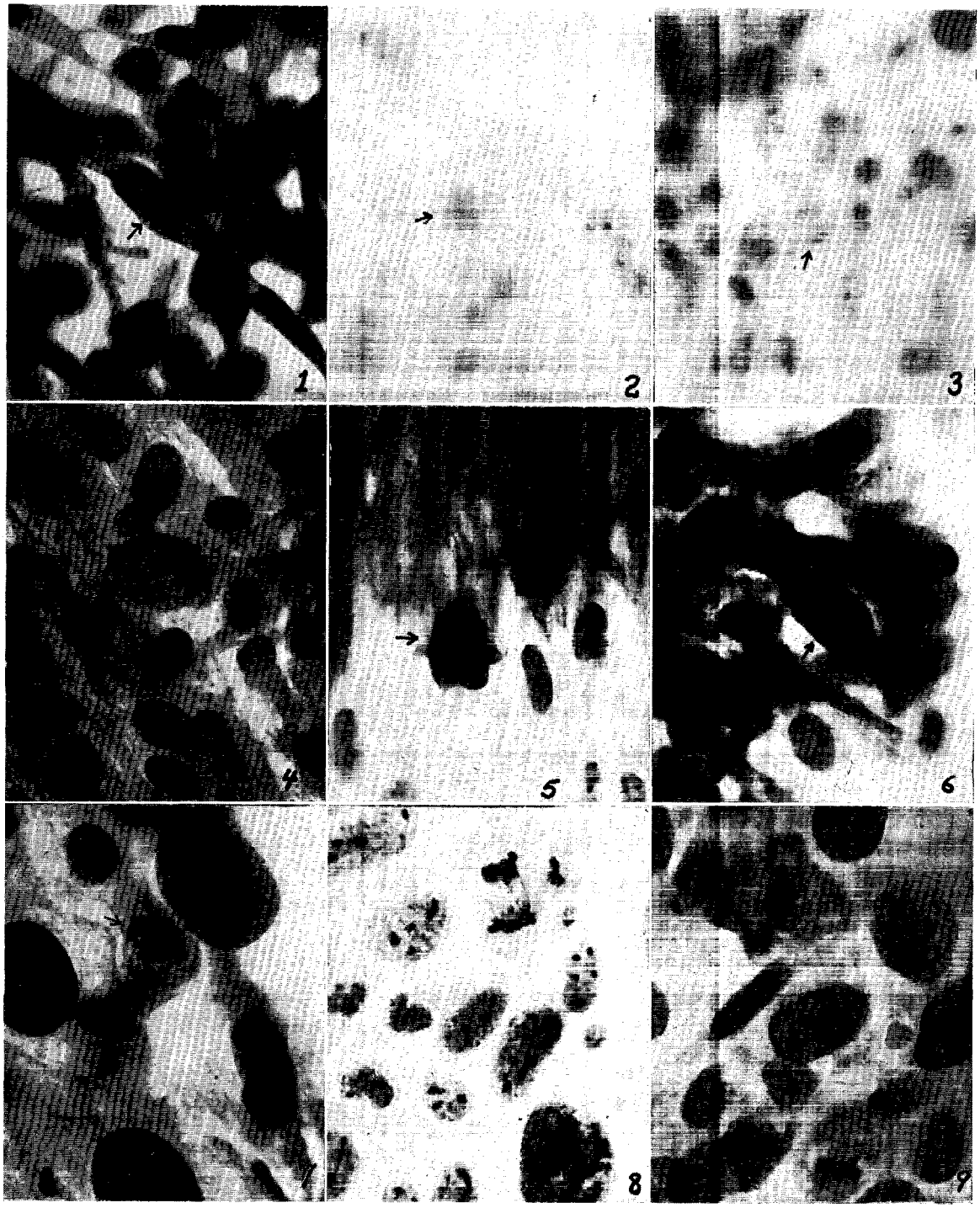

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FIG. 1.—Mouse Sarcoma 180 culture after alkaline phosphatase reaction with muscle adenylic acid, 0.2 mM/1. Metaphase at arrow. Mag. ×600.

FIG. 2.—Sarcoma 180 culture after alkaline phosphatase reaction with yeast adenylic acid, 0.2 mM/1. An early anaphase, not discernible, is at arrow. Mag. ×600.

FIG. 3.—Sarcoma 180 culture after alkaline phosphatase reaction with sodium glycerophosphate, 0.2 mM/1. Anaphase at arrow. Mag. ×600. Photographic treatment was identical with that of Figures 1 and 2.

FIG. 4.—AKm mouse embryo heart culture on same coverslip as Sarcoma 180 culture of Figure 1, after alkaline phosphatase reaction with muscle adenylic acid, 0.2 mM/1. Note anaphase. Mag. ×600.

FIG. 5.—AKm mouse embryo heart culture on same coverslip as Sarcoma 180 culture of Figure 2, after alkaline phosphatase reaction with yeast adenylic acid, 0.2 mM/1. Metaphase at arrow. Mag. ×600.

FIG. 6.—AKm mouse embryo heart culture on same coverslip as Sarcoma 180 culture of Figure 3, after alkaline phosphatase reaction with sodium glycerophosphate, 0.2 mM/1. Anaphase at arrow. Photographic treatment was the same as for Figures 1–5. Mag. ×600.

FIG. 7.—Mouse Carcinoma 1025 culture after alkaline phosphatase reaction with muscle adenylic acid, 1.0 mM/1. Metaphase at arrow. Mag. ×600.

FIG. 8.—Mouse Carcinoma 1025 culture after alkaline phosphatase reaction with yeast adenylic acid, 1.0 mM/1. Note tripolar telophase. Mag. ×600.

FIG. 9.—Carcinoma 1025 culture after alkaline phosphatase reaction with sodium glycerophosphate, 1.0 mM/1. Note tripolar telophase. Photographic treatment was the same as for Figures 7 and 8. Mag. ×600.

FIG. 10.—AKm mouse embryo skin culture after phosphatase reaction with muscle adenylic acid, 1.0 mM/1. Note anaphase. Mag. ×600.

FIG. 11.—AKm mouse embryo skin culture after phosphatase reaction with yeast adenylic acid, 1.0 mM/1. Note anaphase. Mag. ×600.

FIG. 12.—AKm mouse embryo skin culture after phosphatase reaction with sodium glycerophosphate, 1.0 mM/1. Prometaphase at arrow. Mag. ×600. Photographic treatment was similar for Figures 10, 11, and 12.

FIG. 13.—Chick embryo skeletal muscle culture after phosphatase reaction with muscle adenylic acid, 1.0 mM/1. Telophase at arrow. Mag. ×600.

FIG. 14.—Chick embryo skeletal muscle culture after phosphatase reaction with yeast adenylic acid, 1.0 mM/1. Metaphase at arrow. Mag. ×600.

FIG. 15.—Chick embryo skeletal muscle culture after phosphatase reaction with sodium glycerophosphate, 1.0 mM/1. Metaphase at arrow. Photographic treatment was the same for Figures 13, 14, and 15. Mag. ×600.

FIG. 16.—Rous sarcoma cultured from chick embryo, after phosphatase reaction with muscle adenylic acid, 1.0 mM/1. Mitosis at arrow. Mag. ×600.

FIG. 17.—Rous sarcoma culture after phosphatase reaction with yeast adenylic acid, 1.0 mM/1. Mitosis at arrow. Mag. ×600.

FIG. 18.—Rous sarcoma culture after phosphatase reaction with sodium glycerophosphate, 1.0 mM/1. Photographic treatment was the same for Figures 16, 17, and 18. Mag. ×600.

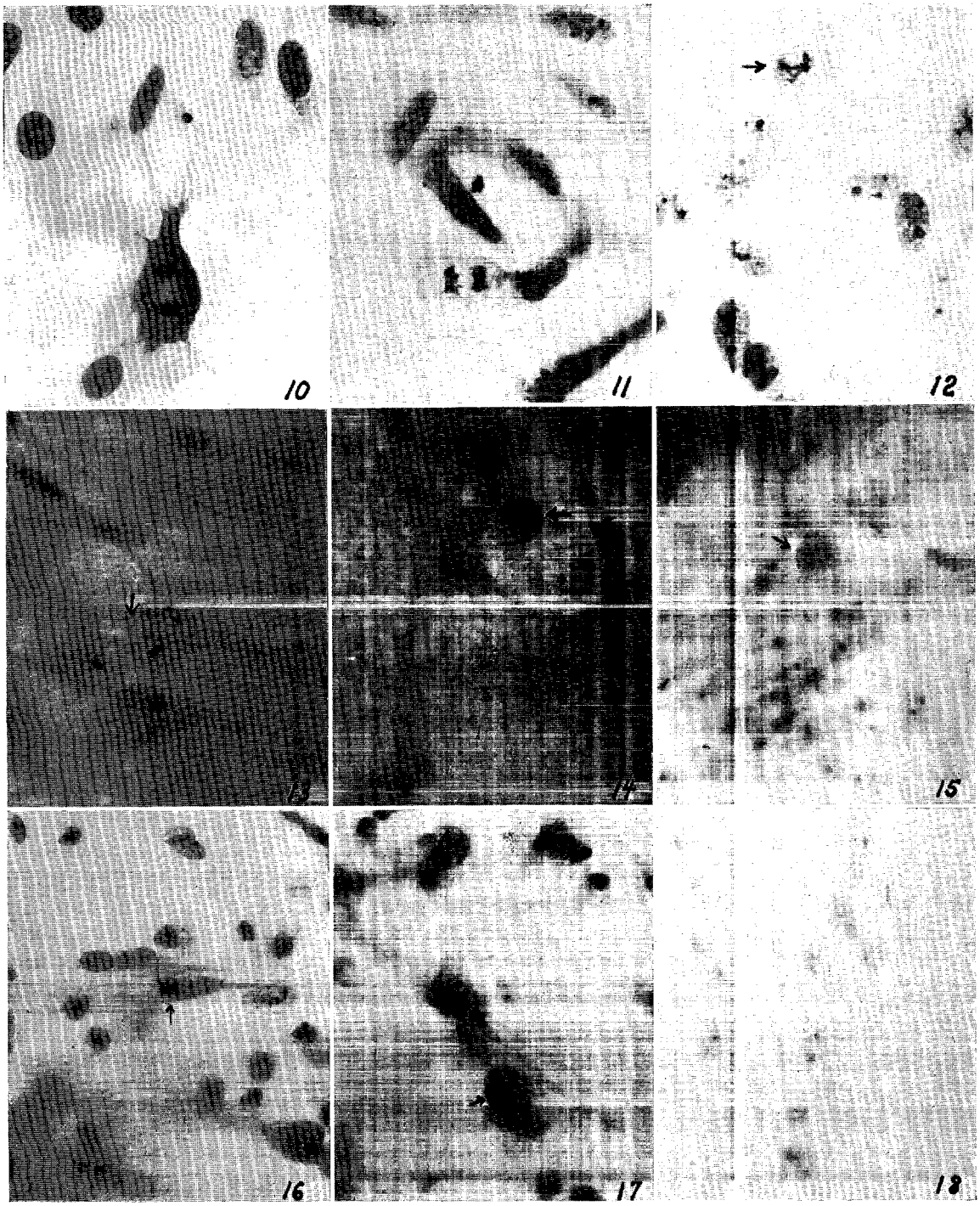

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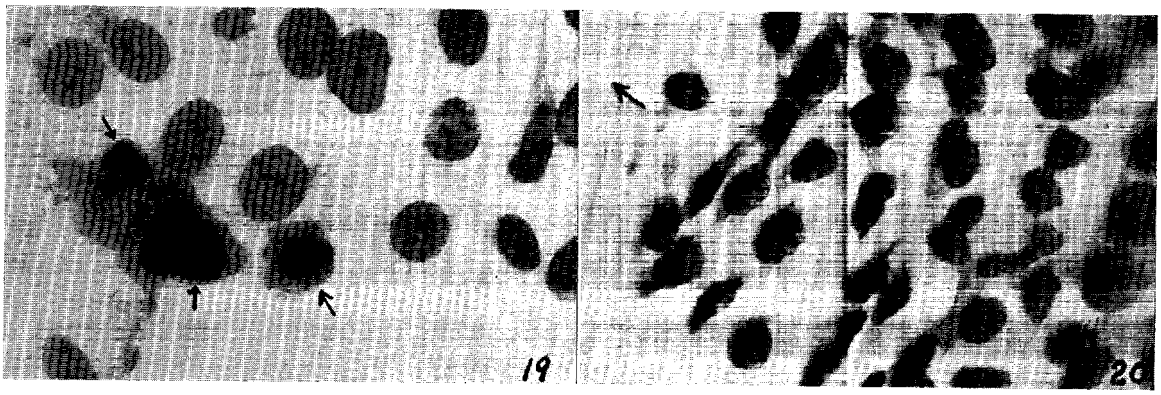
FIG. 19.—Sarcoma 180 cells (arrows) set off from cells of embryonic mouse skin, among which they had wandered, by greater blackening after alkaline phosphatase reaction with 0.2 mM/l muscle adenylic acid. Mag. ×600.

FIG. 20.—Fixation artifact in nuclei of AKm mouse skin cells lying beneath original explant (now removed) when fixed. Arrow points toward center of original explant and in direction of presumed fixative movement. Phosphatase-active material seems to have been halted in its movement by the nuclear membrane. The cells at right edge were not beneath the explant. Muscle adenylic acid, 0.2 mM/l. Mag. ×600.



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galvanometer. Possibly this situation might have been improved if a translucent dye had been formed, instead of an opaque precipitate, as indicator of enzyme activity (8).

The biochemical studies of 5-nucleotidase (6, 11, 12) have been followed by publications in histochemistry indicating that such an enzyme is an entity separate from nonspecific alkaline phosphomonoesterase (5, 9). Our present studies support this view, by and large, with the most cogent evidence being provided by the greater splitting of muscle adenylic acid by fixed cells of Sarcoma 180.

The differentiation with respect to favored substrate cuts across groupings of neoplastic and embryonic cells. It appears that it is not possible to separate normal from malignant cells on the basis of substrate specificity (so far as tested) of the enzyme or enzymes demonstrated by means of the histochemical reaction for alkaline phosphatase.

SUMMARY

1. Fixed cultures of various embryonic and malignant tissues of the mouse, rat, and fowl have been subjected to histochemical tests for alkaline phosphatase.

2. The organic phosphates used as substrates in these tests were muscle adenylic acid, yeast adenylic acid, and sodium glycerophosphate.

3. The tissues differed from one another in the extent of blackening in the resting nucleus (nucleolus) with the various substrates.

4. Mitotic chromosomes gave results similar to those obtained with resting nuclei.

5. Crocker mouse sarcoma 180 cells were outstanding in their high dephosphorylation of muscle adenylic acid and near failure to split yeast adenylic acid and glycerophosphate.

6. Substrate specificity of alkaline phosphatase (or relative activities of nonspecific alkaline phosphatase and 5-nucleotidase) did not serve to distinguish all malignant cells from all normal cells.

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Influence of Diet on the Relative Incidence of Eye, Mammary, Ear-Duct, and Liver Tumors in Rats Fed 2-Acetylaminofluorene*

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INTRODUCTION

In 1948 it was reported from this laboratory (4) that the feeding of 2-acetylaminofluorene to weanling female rats on a semi-synthetic diet produced a 100 per cent incidence of mammary tumors—a much higher incidence than had been reported by other investigators. Following these observations, studies were initiated to determine whether or not modification of the diet would influence the tumor-inducing action of this chemical. It has already been reported that varying the riboflavin content of the diet had no influence on mammary tumor induction and that the inclusion in the diet of a synthetic detergent, or of teropterin, slightly enhanced the rate of tumor formation (5). More recently, results were obtained indicating that high levels of dietary protein were protective against the induction of mammary, ear-duct, and liver tumors by 2-acetylaminofluorene. The omission of fat from the diet markedly inhibited mammary tumor induction but promoted the induction of tumors of the eye (2, 3). It is the purpose of this report to describe the eye tumor, the dietary conditions that promote its induction, and to present data that emphasize the importance of growth rate for the induction of mammary tumors by 2-acetylaminofluorene.

MATERIALS AND METHODS

Weanling female rats of the AES strain were used; they weighed from 35 to 65 gm. and varied from 19 to 23 days in age. The animals were kept in individual screen-bottomed cages; feed and water were supplied daily *ad libitum*, unless otherwise indicated. Records were kept of the amount of feed consumed during the first 16 weeks of the experiment. The diets were prepared at about 20-day intervals and were kept refrigerated.

The composition of the various diets is given in Table 1. Carotene, tocopherol, and calciferol were dissolved in normal hexane; the 2-acetylaminofluorene was dissolved in acetone; these solutions were mixed into the dry ingredients of the diet prior to the addition of fat and the solvents allowed to evaporate. The remainder of the vitamins, in a water solution, and the fats were then mixed into the diet with the aid of a mechanical mixer.

The animals were thoroughly examined for surface tumors once weekly and were usually continued on experiment until they died. At post mortem examination all tumors and other grossly abnormal tissues were preserved and carried through routine procedures for histologic study.

RESULTS

Eye tumors.—Eye tumors were induced with varying frequency in rats on the low fat diets containing 0.03 per cent 2-acetylaminofluorene. The data are summarized in Table 2. Out of a total of 31 rats receiving the various low fat diets *ad libitum*, 10 developed eye tumors—an incidence of 32 per cent. The highest incidence occurred among the rats receiving diet C-98. This diet, which produced eye tumors in six of eleven animals, was sufficiently low in fat to induce a moderate dermatitis indicative of essential fatty acid deficiency near the end of the experimental period of 34 weeks. Eye tumors did not appear in any of the 71 animals receiving the diets containing 15–30 per cent lard (Table 2).

During the course of these studies, it was observed that very poor body weight gains were made by the animals receiving the low fat diets containing 0.03 per cent 2-acetylaminofluorene.

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Average body weights after the 16-week experimental period were only about 100 gm. In contrast, body weights of 175–200 gm. were usually attained in 16 weeks by the animals receiving similar diets containing from 15 to 30 per cent lard. To determine whether or not the development of eye tumors was favored by depressed growth rate, diet C-136 (low fat) and diet C-137 (high fat) were pair-fed, so that the intakes of calories, carcinogen, protein, minerals, and vitamins by the animals on these two diets were equal. Although two of the six animals receiving the low fat diet developed eye tumors, no eye tumors were observed in the six animals on the high fat diet. Despite the equicaloric feeding, the animals on the high fat diet gained about 20 gm. more body weight than those on the low fat diet during the first 16 weeks of the experiment.

The average induction time for the eye tumors was 28 weeks. These tumors originated in the retrobulbar region of the orbital cavity. They were of a firm consistency and usually attained a size of 1–2.5 cm. in diameter in 2–3 weeks after they were first detected. Only one eye was involved in every case. The gross appearance of the eye tumor is shown in Figure 1. It was found to be closely associated with the harderian gland and probably arose from this gland. In some cases the growth of these tumors was so extensive as to occupy completely the orbital cavity with involvement of the eyelid. In such cases the eye was usually completely destroyed. In cases where the

TABLE 1

PERCENTAGE COMPOSITION OF THE DIETS

DIET INGREDIENT	DIET NO. C-5	C-96	C-94	C-98	C-127	C-131	C-136	C-137
Water-extracted casein*	9	9	9	6				
Vitamin-test casein†					12	16	12	16
Degerminated corn grits	20	20	20					
Alcohol-extracted peanut meal‡				30				
Sucrose	50.67	36.67	66.67	59.87	83.17	51.57	83.67	51.56
Salts 5§	4	4	4	4	4	5.3	4	5.3
L-cystine	0.3	0.3	0.3	0.1	0.3	0.4	0.3	0.4
Lard	15	30.0				26		26.7
Cod liver oil	1							
Corn oil					0.5	0.7		
2-Acetylaminofluorene‖	.03	.03	.03	.03	.03	.03	.03	.04
Vitamin supplements								
B-carotene (mg.)		0.5	0.5	0.5	0.5	0.7	0.5	0.7
Calciferol (μg.)		12.5	12.5	12.5	12.5	16.7	12.5	16.7
Vitamin E# (mg.)	5	5	5	5	5	6.7	5	6.7
B-vitamin solution** (ml.)	5	5	5	5	5	6.7	5	6.7

* Commercial casein percolated for 5 days with water acidified with 0.20 per cent acetic acid overnight and finally washed with 95 per cent ethanol and dried.

† General Biochemicals.

‡ Forty-five per cent protein meal percolated for 1 week with hot methanol.

§ J. Nutrition, 33:155–68, 1947.

‖ Obtained from Eastman Kodak Co.

Equal parts of alpha-tocopherol and alpha-tocopherol acetate.

** The B-vitamin solution contained the following in milligrams per 5 milliliters: thiamin and pyridoxin, 0.2 each; riboflavin, 0.4; calcium pantothenate, 1.0; niacin, 2.0; inositol, 20; and choline chloride, 200.

TABLE 2

TUMOR INDUCTION BY 2-ACETYLAMINOFLUORENE IN WEANLING RATS RECEIVING DIETS WITH AND WITHOUT FAT

DIET No.	DIET Fat content (per cent)	ANIMALS No.	Av. body weight Initial (gm.)	Av. body weight 16-week (gm.)	Av. survival time (weeks)	FOOD Av. daily intake per rat (gm.)	TUMORS No. of animals with Mammary	Ear-duct	Liver	Eye
C-5	16	62	47	176	28	6.6	46	29	40	0
C-96	30	3	53	184	27	6.0	3	2	2	0
C-94	0	8	55	105	29	4.8	0	5	7	1
C-98	0	11	51	91	34	5.0	1	5	4	6
C-136*	0	6	48	104	31	5.8	0	6	5	2
C-137†	26	6	44	127	31	4.4	0	5	2	0
C-127	0.5	6	46	114	31	6.6	1	2	5	1
C-131	26	6	46	200	31	7.2	6	3	4	0

* Animals on diet C-136 each received 0.20 ml. of corn oil once weekly as a source of essential fatty acids.

† Animals on diet C-137 were restricted in food intake so that the consumption of total calories, vitamins, minerals, protein, and 2-acetylaminofluorene was equal to that of their litter mates receiving diet C-136.

tumors were smaller, the eye could be dissected free of tumor tissue.

Microscopic studies of slides prepared from the eye tumors revealed that they were carcinomas, although there was considerable variation in the cellular structure (Figs. 2 and 3). Glandlike arrangements suggestive of adenocarcinoma were found in some areas, and in other areas the arrangement was almost suggestive of sarcoma. The prominent cell type was a relatively large round cell with a hyperchromatic nucleus. There were variations to oval and odd-shaped nuclei. The cells were closely packed, with only a small amount of connective tissue stroma. There were numerous mitotic figures. Numerous lymphatic spaces with tumor cells packed closely around them were observed (Fig. 3). Local invasion of muscle tissue is illustrated in Figure 4. Tumor emboli were observed in blood vessels in the orbital region (Fig. 5), and metastases to the lung were observed in two of the ten eye-tumor cases (Fig. 6).

Mammary tumors.—The incidence of mammary tumors was extremely low among the animals receiving the low fat diets. Only 2 of the 31 animals developed this type of tumor (Table 2). In contrast, mammary tumors occurred in 55 of the 71 animals that received the diets containing 15–30 per cent lard. However, when a 26 per cent lard diet (diet C-137) was fed at a restricted level so that poor growth ensued, mammary tumors were completely inhibited. Thus, the poor body growth regularly observed in animals consuming 2-acetylaminofluorene in low fat diets could be responsible for failure of mammary tumors to develop. A relatively rapid growth rate is apparently essential for the uniform induction of mammary tumors by 2-acetylaminofluorene in weanling rats. This is further emphasized by the results obtained with diet C-5 (Table 3). All the 62 animals represented in these data received diet C-5 and served as controls in a series of studies designed to test the importance of various modifications of dietary vitamin levels on mammary tumor induction by 2-acetylaminofluorene. In Table 3, the body weights of these animals at 16 weeks have been grouped in ascending order. The body weights ranged from 144 to 235 gm. It is obvious from the data in Table 3 that the rats which made the more rapid weight gains also more consistently developed mammary tumors. Twenty-six of the 27 rats, weighing 176 gm. or more at 16 weeks, developed mammary tumors. In contrast, only 20 of the 35 rats, weighing 175 gm. or less at 16 weeks, developed mammary tumors.

The variation in weight gain among the rats receiving diet C-5 could not be attributed to differences in initial body weight. The 35 slowly growing rats had an average initial weight of 47 gm. as did the 27 rapidly growing rats. The differences in gain likewise could not be traced to any particular litters. The individual variations in gains exhibited by the data in Table 3 could be attributed directly to variations in total feed consumed.

The average induction time for mammary tumors was 19 weeks. The average induction time for the rats weighing less than 176 gm. at 16 weeks was 20 weeks (range, 12–30 weeks), as compared to an average of 18 weeks (range, 15–27 weeks) for those weighing more than 175 gm. at 16 weeks. However, with the wide variations apparent in both groups, the significance of this difference is questionable.

TABLE 3

INFLUENCE OF WEIGHT GAINS ON TUMOR INDUCTION BY 2-ACETYLAMINOFLUORENE

Body weight			No. of rats with		
Initial (gm.)	16 Weeks (gm.)	No. of rats	Mammary tumor	Ear-duct tumor	Liver tumor
49	155 or less	8	4	4	6
45	156–165	13	4	9	7
47	166–175	14	12	7	10
Subtotal		35	20	20	23
48	176–185	9	9	5	8
45	186–195	6	5	1	3
46	196–205	6	6	2	2
49	206 or more	6	6	1	4
Subtotal		27	26	9	17
Total		62	46	29	40

Other tumors.—Tumors of the ear duct developed in an average of 24 weeks. This type of tumor appeared to develop less frequently under conditions of rapid growth associated with high feed consumption and a high incidence of mammary tumors (Table 3). For example, only 4 of the 18 most rapidly gaining rats on diet C-5 had ear-duct tumors. In contrast, 25 of the 44 more slowly growing rats on this diet developed ear-duct tumors.

The development of liver tumors did not appear to be influenced by changes in dietary fat level. The rate of growth of the animal likewise did not appear to influence the induction of liver tumors. No attempts were made to establish the induction time for internal tumors.

Three unusual types of tumors were observed among the animals receiving diet C-5. One rat had a tumor on the lateral aspect of the buccal cavity, another rat had a kidney tumor, and a third rat had an intestinal tumor.

DISCUSSION

The appearance of eye tumors in rats fed low fat diets and the absence of this type tumor in rats

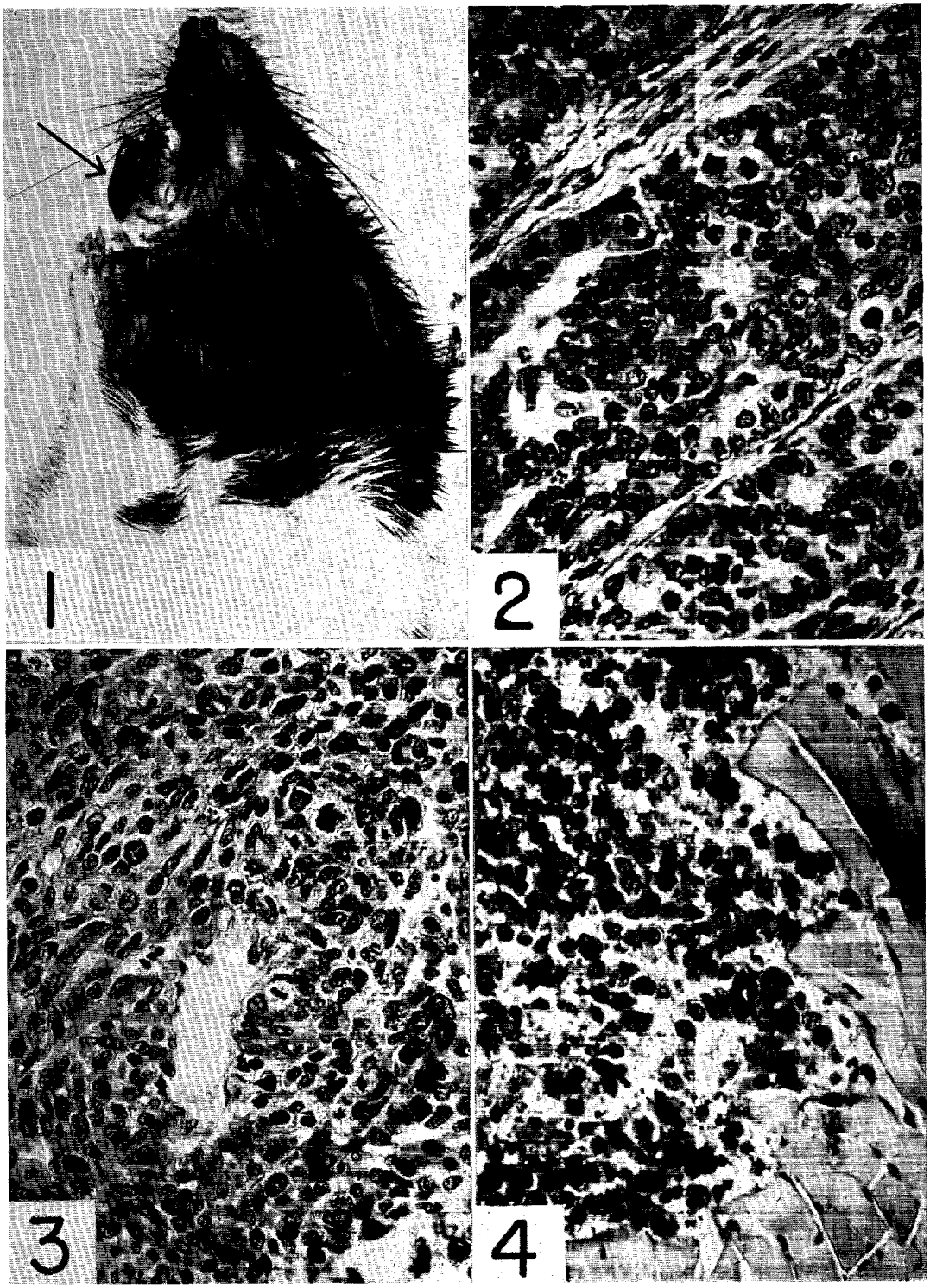

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FIG. 1.—Photograph showing the gross appearance of a tumor of the orbital cavity of a rat fed a low fat diet containing 0.03 per cent 2-acetylaminofluorene.

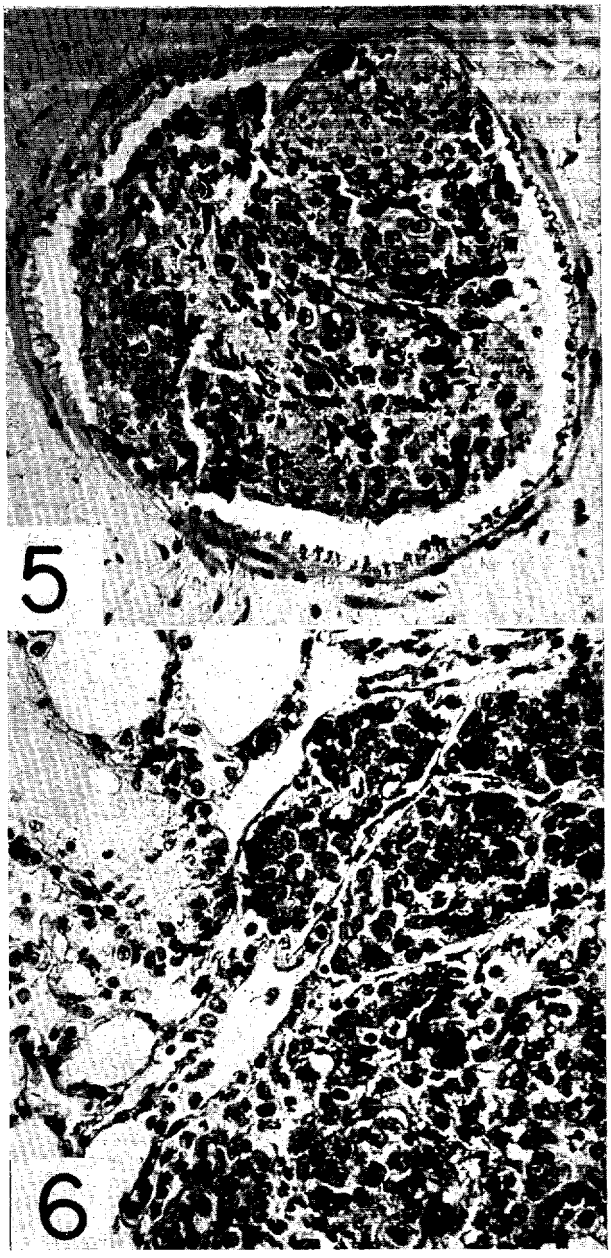
FIG. 2.—Photomicrograph illustrating the general histology of a tumor of the orbital cavity (harderian gland). Note the "glandlike" arrangement of the cells and the numerous mitotic figures. Hematoxylin and eosin. ×375.

FIG. 3.—Photomicrograph of a section from another area of the tumor showing closely packed tumor cells around a lymph vessel. Hematoxylin and eosin. ×350.

FIG. 4.—Photomicrograph of a section of the same tumor to illustrate the local invasion of muscle tissue by the tumor cells. Hematoxylin and eosin. ×375.

FIG. 5.—Photomicrograph of a section through a blood vessel which contains a tumor embolus in the orbital region of the rat shown in Figure 1. Hematoxylin and eosin. ×325.

FIG. 6.—Photomicrograph of a section of lung from the rat shown in Figure 1 illustrating metastasis of the orbital tumor (harderian gland tumor) to the lung. Hematoxylin and eosin. ×325.



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receiving similar diets with fat indicate that the site of action of 2-acetylaminofluorene in the animal body can be influenced by diet. Eye tumors induced by feeding 2-acetylaminofluorene apparently have been observed only rarely by other investigators using this carcinogen. Bielschowsky (1) reported a cancer of the eyelid in one animal in a series of studies which included several hundred animals. A detailed description of this tumor was not given. Morris and associates (6) reported a transplantable tumor involving the harderian gland, which was originally induced by feeding 2-acetylaminofluorene and thiouracil. The type of diet fed was not mentioned in either of these reports. That tumors can be produced in the eye of rats by 2-acetylaminofluorene should perhaps be expected in view of the results of Weisburger and associates (8). These workers observed that the harderian gland accumulated appreciable quantities of this carcinogen after the C^{14}-labeled compound was administered to rats.

One of the requirements for mammary tumor induction by 2-acetylaminofluorene in rats appears to be that the animal must grow at a normal or nearly normal rate. Underfeeding and caloric restriction are known to inhibit the induction of various types of spontaneous and induced tumors (7). A lowered caloric intake, probably due to the presence of the carcinogen in the diet, explains the lowered incidence of mammary tumors in slowly growing rats in the present study. Furthermore, a diet known to produce a high incidence of mammary tumors when fed *ad libitum* produced no mammary tumors when fed at a restricted level. The ear-duct tumor incidence appeared to decrease when diets were used that promoted rapid growth (high caloric intake) and produced a high incidence of mammary tumors. A high caloric intake did not appear to be essential for the induction of either ear-duct or liver tumors.

The present results thus indicate that the type of diet fed and the quantity consumed can have a very definite influence upon the site of action of 2-acetylaminofluorene.

SUMMARY

Eye tumors, presumably originating in the harderian gland, were induced in 10 of 31 weanling rats fed 0.03 per cent 2-acetylaminofluorene on low fat diets for 7–8 months. Rats fed such diets made very poor weight gains. Attempts to induce eye tumors by feeding fat-containing diets *ad libitum* or at a restricted level of caloric intake were unsuccessful. Whether or not fat was present in the diet, mammary tumor induction was greatly decreased when the animals made poor weight gains. Conditions that favored mammary tumor induction, rapid growth, and high caloric intake appeared to decrease the induction of ear-duct tumors. The type or quantity of diet consumed had little or no influence upon the induction of liver tumors. The results emphasize that the site of carcinogenic action of 2-acetylaminofluorene can be markedly influenced by the type and quantity of diet fed.

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Spontaneous Thyroidal Tumors in the Swordtail *Xiphophorus montezumae**

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Early studies of the thyroid tumors of trout (3–5) have demonstrated the relationship between iodine supply and hyperplastic growth of thyroid cells. Experimental work with fishes provided much of the background for the present understanding of the etiology of human goiters. In contrast with encapsulated mammalian goiters, however, hyperplastic thyroids in fishes are remarkable for their invasive reaction to surrounding tissues. This apparently invasive property of unencapsulated fish thyroidal cells and follicles led earlier workers to consider the glandular tumors as "carcinomas" (1). However, later work showed that the regression of thyroidal tumors in trout could be induced by adding iodine to the water in which the fish were maintained (3).

Spontaneous thyroidal tumors have been reported in at least eighteen species of fresh-water and marine fishes. Like trout, all of them have been observed under cultivation in aquaria or in fish hatcheries (6). While the goiters in fresh-water forms are often caused by a low iodine supply in the water or food, this explanation hardly seems applicable for salt-water species which live in a relatively iodine-rich environment and which feed upon such iodine-rich foods as ocean shrimp and clams (6). The morphologies of thyroidal tumors in fishes are all quite similar.

In this report we propose to describe a spontaneous thyroidal tumor of high incidence in a species of Mexican fresh-water fish, the swordtail, *Xiphophorus montezumae*. Our knowledge of the environmental conditions under which these tumors develop makes it possible to suggest certain generalizations concerning their natural history and genetics. In addition, we have attempted to obtain an estimate of the degree of dysfunction of the tumorous thyroid tissue by means of the ability of these fish to utilize radioactive iodine. This was recorded autographically.

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METHODS

The original stock of *Xiphophorus montezumae* was obtained in 1939 from the headwaters of the Rio Axtla in the Mexican state of San Luis Potosi. Laboratory-bred stocks were propagated in 5-, 10-, or 20-gallon aquaria. These contained plants of the genera Nitella, Cryptochoryne, and Sagittaria, the rooted aquatics being planted in clean, coarse gravel. The overhead daylight illumination was filtered through the glass of the skylight. The water in the aquaria was originally obtained from the common New York City supply. Once utilized for aquarium purpose the water has been used over and over again for 10 years. From time to time aerated tap water is added to a common reservoir of the conditioned water. Other closely related fish species were kept in the laboratory under virtually identical conditions and management including the following: two species of Xiphophorus, *X. pygmaeus* and *X. hellerii;* and four species of Platypoecilus, *P. maculatus*, *P. xiphidium*, *P. variatus* and *P. couchianus*. A small colony of *Lebistes reticulatus* was maintained. All fishes were fed the same diet: a fresh-liver-Pablum-cereal-mixture (2), live tubificid worms, and dried, shredded ocean shrimp. In addition, particularly, during the first 2 months of life, the fishes were given live Daphnia and Aulophorus.

After eight generations of rather close inbreeding (but not by brother-sister mating) numerous montezuma swordtails (*X. montezumae*) developed pronounced pinkish swellings in the region of the isthmus (i.e., between the bones of the lower jaw) (Fig. 1).

A number of montezuma swordtails with externally visible thyroidal tumors were fixed. Others were isolated and kept under close observation. Fixation in Bouin's fluid made special decalcifica-

tion procedures unnecessary. The lower jaws and the adjacent region of fourteen of these fish, some with and some without tumors, were serially sectioned for detailed microscopic study.

Eight tumor-bearing animals were placed in 1-liter aquaria containing 25–50 μc. of carrier-free radioiodine, I^{131}, for 24 hours. The serial sections of their entire lower jaws were exposed to squares of No-screen x-ray film, and radioautographs were prepared from them.

Morphology of thyroidal growths.—Gross examination of mid-sagittal sections revealed the anterior and posterior limits of the tumors which measured about 4 × 5 mm. Anteriorly, the thyroid was always only slightly forward of the gill-bearing visceral arches. Posteriorly, masses of thyroid tissue surrounded the base of the ventral aorta and the bulbar portion of the heart, extending almost as far back as the auricle. The tongue, which was much enlarged and consisted almost entirely of thyroid tissue (Fig. 3), extended dorsally nearly to the roof of the mouth. Ventrally, the thyroid tissue grew through the muscular mass of the lower jaw, between the elements of the visceral skeleton, and formed a nodular bulge clearly visible on the lower side of the head at the isthmus. Externally this characteristic swelling appeared pink and made identification of thyroid-tumorous fish an easy task. Laterally, thyroid tissue spread into the gills and to the bases of the opercula. The fairly loose connective tissue appeared to offer little resistance to the spread of the growth.

Penetration or invasion of the walls of the large aortic branches was not seen. However, the large masses of thyroidal tissue, apparently under some pressure due to expansive growth, enveloped the bulbar heart, the aorta, and aortic branches of the gills, probably impairing the efficiency of the vascular channels and thus interfering with the respiratory demands of the body.

In addition, tumor tissue penetrated the gills from below. The degree of gill involvement in various fish appeared roughly proportional to the size of the tumor growth. The tumor might, therefore, be regarded as a progressive type. In spreading into a gill the proliferating thyroid tumor cells caused the epithelium to pull away from its skeletal and vascular elements. The involved secondary gill filaments, enlarged by the tumor tissue, were found in various stages of pressure against one another and, finally, coalescence. Nests of gill epithelium were thus buried within thyroidal tumor masses. The epithelial cells in such nests were pyknotic. The available respiratory epithelium was radically reduced in this way, and it is probable that the expanding tumor growth imposed a real physiological burden upon the fish, destroying the efficiency of the gills as respiratory organs. This may contribute greatly, if not to a major extent, to the death of the tumor-bearing fish.

The tumors, though unencapsulated, appeared surprisingly regular at their peripheries, especially along the posterior border. Elsewhere, broad or narrow tongues of tumor tissue, arising from the main mass, invaded normal areas of the body. The penetration of muscle appeared never to be direct, but the invasion followed the endomysial connective tissue septa. In the process the muscles were divided into successively smaller units and finally isolated into individual muscle fibers. Such fibers appeared to undergo shrinkage and loss of cross striations. Seemingly, they finally degenerated completely, for only a few of the isolated muscle fibers were found imbedded in the main tumor mass.

Completely separated elements of the visceral skeleton also were found buried, and in a degenerative state, within the thyroid tumor (Figs. 2 and 3). Most cartilage and bone cells were pyknotic. Thyroid cells almost always were found within bone cavities. Eroded fenestrae in the bones (Fig. 2) were found, through which entry of the thyroidal tissue was apparently gained. Many tumor-imbedded fragments of bone were greatly reduced in size and abnormal in shape, giving further evidence of erosion. This relation between bone and the thyroid tumor implies that either (*a*) the bone undergoes some sort of autolytic change when isolated in this way, or (*b*) the thyroidal tissue itself has an erosive effect on bone.

The microscopic structure of the hypertrophied thyroid tissue was not uniform. Although the main mass of thyroid was microfollicular or afollicular, there was considerable variation in cell size and colloid content (Fig. 2). Afollicular areas of thyroid were usually located in the medullary portion of the tumor. However, in areas where penetration of neighboring tissues was in progress, especially in the gills, afollicular cells were found at the periphery. The individual cells were larger than in normal thyroidal tissue, and even though no follicular organization was obvious, the nuclei in most instances were eccentric in position. Frequently the cytoplasm was somewhat vacuolated and contained cosinophilic droplets of colloid-like substance.

Microfollicular areas in the tumor merged with the afollicular portions. The smallest follicles in such areas were less than 20 μ in diameter. Each consisted of a few cells grouped about a small droplet of colloid, or about a tiny empty lumen. The

constituent cells of microfollicular portions of the tumor were quite similar to those of the afollicular parts and they appeared to be virtually as "invasive." They, too, were found within bones and in the gills.

In the tongue region the tumorous tissue frequently was less dense than in other parts. Here edematous spaces clearly separated the small follicles or individual cells. Some of the spaces were endothelium-lined, and, hence, seemed to be enlarged lymph channels. Seemingly in response to freedom from immediate pressure of neighboring cells, there was a rounding up of the cells within a small area. Under these circumstances the rounded cells of an afollicular thyroid region resembled young adipose tissue of the "brown fat" type.

There were relatively few follicles of approximately normal size, morphology, and colloid content. They occurred in groups either near the edges of the thyroidal growths, or especially near their posterior borders (Fig. 2).

Cystic follicles were found in the tumor of one animal only. The lumina of the cystic area, which was at the anteroventral end of the tumor, were irregular in shape, and some were in communication with one another through a series of gaps. The epithelium was cuboidal, and the lumen contained a scanty eosinophilic material, stringy in texture. The general histology of this cystic area resembled that of the mammalian seminal vesicle.

A few hemorrhages were found within the thyroidal tumors. They usually were associated with numerous pigment-laden macrophages.

Radioautographs.—The radioautographs of all afollicular, microfollicular, and cystic areas of the tumors were very weak, indicating that iodine accumulation in these areas was hardly, if at all, greater than in nonthyroidal areas (Fig. 3). This is in contrast to the behavior of thyroid tissue in unaffected fishes. However, evidence of approximately normal intensity of iodine storage was seen on most radioautographs in small spots at the periphery of the tumors. These dark autographs were found to have been made by the relatively few apparently normal follicles still present in the thyroidal growths.

DISCUSSION

In morphological features, at least, the thyroidal tumor of *Xiphophorus montezumae* is quite similar to the thyroidal "carcinomas" of trout and similar tumors of other fishes. The similarity extends, furthermore, to the fact that the tumors developed in a group of captive animals. We have no information concerning the incidence of such tumors under natural situations, except that among hundreds of specimens collected in the field from various tributaries of the Rio Axtla, no externally visible thyroidal tumors were seen.

One seemingly significant difference between the tumors of *X. montezumae* swordtails and those of trout is in respect to the age at which they develop. The thyroidal tumors of trout were relatively large in young animals. They diminished or even regressed completely (3) in older trout, if the animal survived beyond a critical point. In *X. montezumae* the growths did not appear until the animals had reached almost adult size, and they increased in size until the death of the diseased fish.

Some of the circumstances attending the development of the tumors seem worthy of consideration, since they further define the character of the growths. While the thyroidal tumors of trout appear to form in goitrogenic response to a low concentration of iodine in the environment (3, 6), this does not appear to apply to *X. montezumae*. Dried ocean shrimp, which composed a large part of the diet of our aquarium-reared fishes, have a high content of iodine and actually had a curative effect upon the thyroidal tumors of trout studied by Marine (3). It is conceivable that our laboratory stock of *X. montezumae* has an extremely high iodine requirement—one which exceeds its dietary supply.

In our laboratory the following species kept under the identical dietary and environmental conditions develop thyroidal tumors very rarely: *Xiphophorus pygmaeus*, *Platypoecilus maculatus*, and *P. xiphidium*. The tumors have never been found in *Xiphophorus hellerii*, *Platypoecilus variatus*, or *Platypoecilus couchianus*. In nature, *X. montezumae*, *X. pygmaeus*, and *P. variatus* are found living together occasionally in the Rio Axtla at Axtla, San Luis Potosi, Mexico. *X. montezumae*'s greater susceptibility, whether or not it is due to a higher exogenous iodine requirement, may, therefore, be mediated by a specific genetic difference.

SUMMARY

In a laboratory population of swordtails (*Xiphophorus montezumae*) obtained originally in 1939 from the Rio Axtla in Mexico, a high incidence of spontaneous thyroidal tumors has been observed. In two other species of Xiphophorus, four species of Platypoecilus, and in *Lebistes reticulatus*, kept under identical conditions, thyroidal tumors are very rare or are never seen.

The thyroidal tumor is not uniform in microscopic structure, consisting of areas of normal follicles, very small follicles, nonfollicular cell masses, and a few cystic follicles. It has the property of

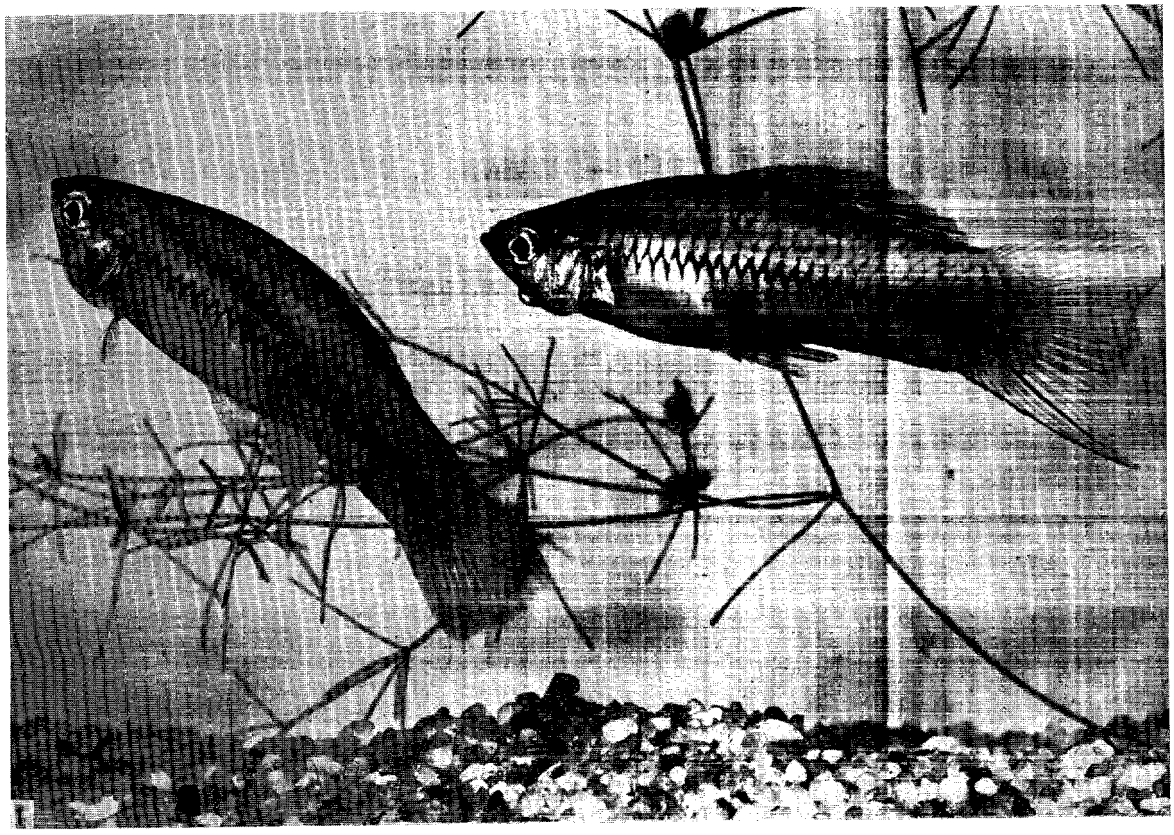

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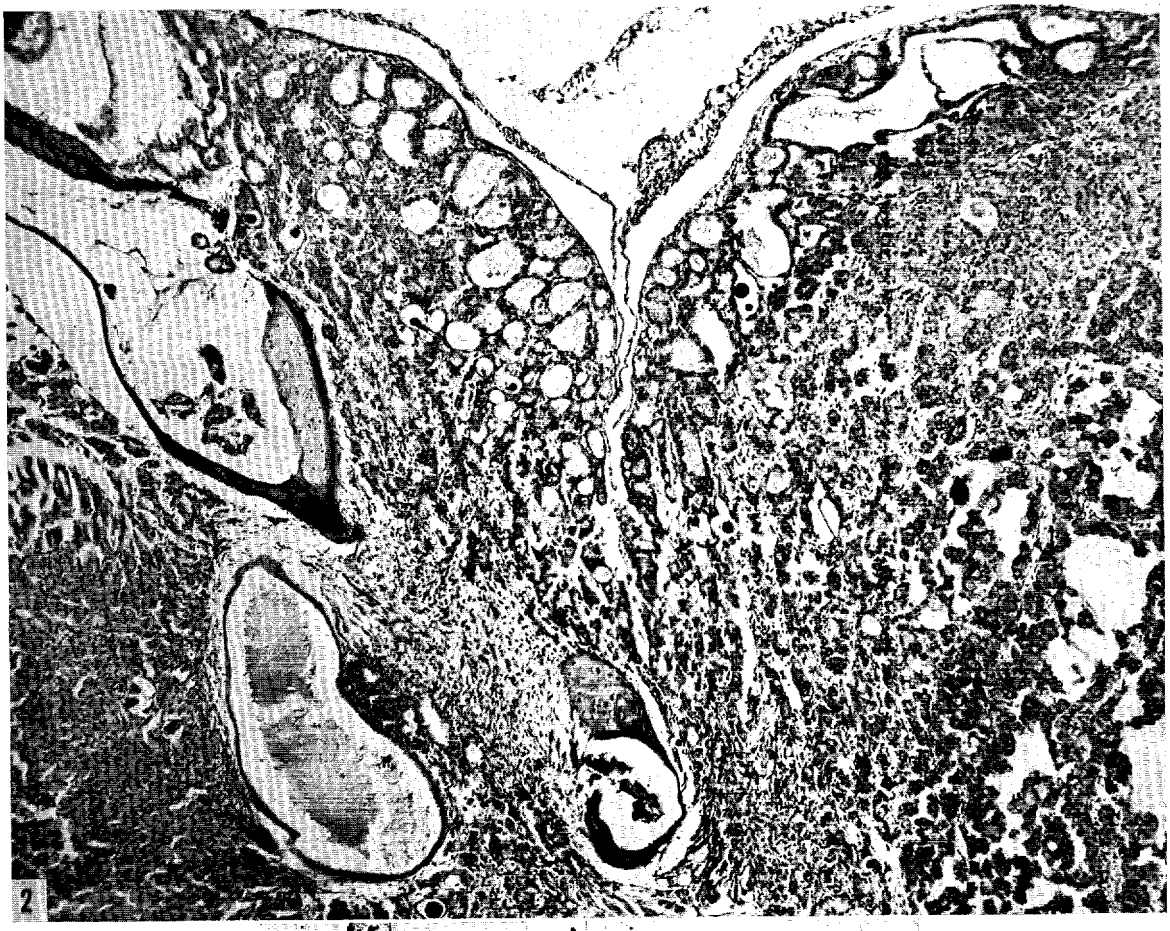
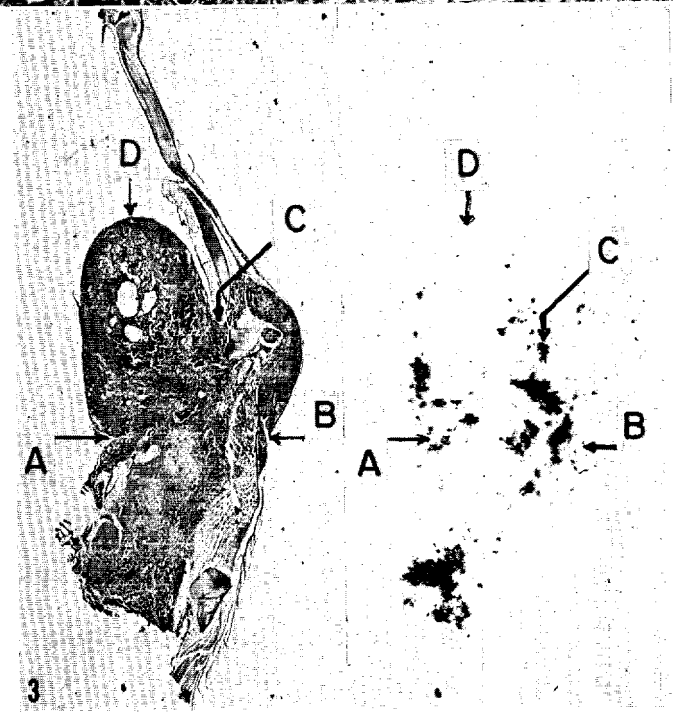
FIG. 1.—Two adult *Xiphophorus montezumae*, a female on the left, a male on the right. Both have thyroidal tumors. The tumor of the male is visible as an outgrowth below the jaw. In both the opercula are lifted away from the head due to the infiltration and thickening of their bases.

FIG. 2.—Section of thyroidal tumor. All normal tissue beneath the mucosal epithelium (top) has been supplanted by thyroidal tissue. Two bones are visible in this section (middle and left). Both have been perforated, and contain thyroid. The only follicles which metabolize radioiodine (revealed by autograph in Fig. 3) are the large ones at the top edge of the growth, just beneath the mucosal epithelium (near point *A* in Fig. 3). A few degenerate muscle fibers are visible as very dark spots about the diameter of a small thyroid follicle. The large blood vessels are branchial vessels. The remainder of the growth is mostly microfollicular, with several afollicular areas (near center).

FIG. 3.—Section of the entire lower jaw (left) of the same animal as in Figure 2, and the radioautograph (right) made by this section. In the photomicrographs of both the section and the autograph, corresponding points *A*, *B*, *C*, and *D* have been placed for reference. The area *A-C-D* is the much enlarged tongue. The area to the right of the line *B-C* is the portion of the tumor which projects visibly externally as a dimple on the lower side of the jaw (see Fig. 1). Most of the growth does not metabolize iodine normally. A few follicles at or near the edge of the growth produce a normal autograph. Parallel and posterior to (below) the line *A-C* are several bones and bases of gill arches.





invading bone. By gradually filling the visceral arches it apparently interferes with respiration, contributing finally to the death of the fish.

In radioautographs of thyroidal tumors of swordtails given radioactive iodine it appears that the few normal follicles are normally functional in respect to iodine metabolism. The remaining areas of the tumor, on the other hand, seem to lack this ability to a large extent.

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The Distribution and Excretion of Stilbamidine-C^{14} in Mice

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A number of diamidines, of which Stilbamidine (stilbene-4, 4′-dicarboxamidine) has been the most widely employed, have been found useful in the treatment of trypanosomiasis, leishmaniasis, and infected wounds in humans, and of babesiasis in animals. In addition, some indication of antimalarial activity in birds and monkeys has been obtained. These applications have been reviewed by Rosenberg (12). In 1946 Snapper introduced the use of Stilbamidine in the treatment of multiple myeloma. In many patients the drug causes relief of bone pain. In the cytoplasm of the plasma cells which are responsible for this malignant neoplastic disease, there is a deposition of granules containing a Stilbamidine salt of ribonucleic acid; and this is particularly true in those patients with high serum globulin. The nucleic acid is thought (13) to have been displaced from cytoplasmic nucleoprotein by Stilbamidine, and Kopac (11) has demonstrated the occurrence of such a dissociation of protamine nucleate by Stilbamidine *in vitro*. The granules have not been observed in the other cellular components of the marrow of patients treated with the drug, and this fact has led to the suggestion (13) that the plasma cells contain nucleoprotein which is different from that of normal cells in that it is more readily dissociated by Stilbamidine. If it is true that the uptake of Stilbamidine by the plasma cells is greater than that by other cells, and if the differential is sufficiently great, it should be possible to irradiate selectively the plasma cells by the administration of Stilbamidine containing a sufficiently high content of a radioactive isotope.

This paper describes the synthesis of Stilbamidine containing carbon 14 and a study of its distribution and excretion in mice. These experiments are preliminary to others aimed at determining whether selective irradiation can be achieved by administration of the radioactive drug to patients with multiple myeloma. The Stilbamidine, synthesized by the reaction sequence shown in Chart 1, was prepared as the salt of isethionic acid, which, because of its easy solubility, is the form in which the drug is customarily administered.

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PROCEDURE

Synthesis of Radioactive Stilbamidine Diisethionate

Isethionic acid.—Barium isethionate was prepared from diethyl sulfate and fuming sulfuric acid (6). An aqueous solution of the salt was exactly neutralized with sulfuric acid, and after removal of barium sulfate the solvent was evaporated *in vacuo* at room temperature. The white, highly deliquescent residue was dried in high vacuum.

Sodium cyanide-C^{14}.—Isotopic sodium cyanide was prepared as an aqueous solution containing excess sodium hydroxide by reduction of 10 mc. of radioactive barium carbonate with sodium azide (1). The yield was 60 per cent.

Cuprous cyanide-C^{14}.—Radioactive cuprous cyanide was prepared from cupric sulfate and isotopic sodium cyanide in the presence of bisulfite. Bisulfite serves to reduce the cupric ion (3); without it the cupric ion is reduced at the expense of cyanide, half of which is lost as cyanogen. The reaction vessel was a 40-ml. conical Pyrex centrifuge tube, fitted with a three-hole rubber stopper. Through the respective holes were inserted a sealed stirrer, the stem of a small separatory funnel, and a gas outlet tube. The outlet tube was connected to an absorber containing alkaline permanganate to catch any cyanogen which might be formed. Into the centrifuge tube was placed a solution of 1.50 gm. (6.00 mM) of cupric sulfate pentahydrate in 10 ml. of water acidified with 3 drops of 6 M sulfuric acid. The separatory funnel received 15 ml. of a solution containing 3.85 mM of radioactive sodium cyanide and 1.00 gm. (3.97 mM) of sodium sulfite heptahydrate. The solution was about 0.5 M in sodium hydroxide. A drop of phenolphthalein was added to this solution, followed by enough 6 M sulfuric acid to discharge the pink color. The solution was added dropwise to the cupric sulfate while the mixture was stirred vigor-

ously. A white precipitate of cuprous cyanide formed rapidly, and after 15 minutes a current of air was drawn through the apparatus to carry any gaseous products into the permanganate. The cuprous cyanide was collected by centrifugation, washed with water, and vacuum-dried. The yield was 0.350 gm., 100 per cent based on cyanide. The yield in this step was ordinarily about 80 per cent. The product was not removed from the centrifuge tube, which was used as the reaction vessel in the next step.

4,4′-Dicyano-C^{14}-stilbene.—The dinitrile was prepared by reaction between radioactive cuprous cyanide and 4,4′-dibromostilbene (2). To the cuprous cyanide was added 665 mg. (1.95 mM) of dibromostilbene, and the solids were mixed with a porcelain spatula. One ml. of dry pyridine was then stirred in, and the mixture was refluxed 1.5 hours (bath temperature 190°–210°). The mixture was cooled somewhat, 2 ml. more pyridine was added, and the mixture was refluxed 5 minutes longer. It was then cooled almost to the point of solidification, and 9 ml. of concentrated hydrochloric acid was added. Lumps were broken up by thorough stirring, and the mixture was centrifuged. After three washes with 2-ml. portions of concentrated hydrochloric acid, followed by three 2-ml. portions of water, 471 mg. of pale yellow product was obtained, melting at 265°–272°. This material was used in the next step without further purification.

Conversion of the nitrile to the imidoester.—The dicyanostilbene was transferred to a 15 × 150 mm. Pyrex ignition tube which was then constricted near the mouth. Water introduced by the glass-blowing lamp was removed by attaching the tube to a high vacuum line for 5 minutes. To the contents of the tube were then added 5 ml. of C. P. chloroform and 0.75 ml. of C. P. methanol (2). The mixture was saturated at 0° with dry hydrogen chloride, and the tube was sealed. It was then rotated mechanically for 60 hours at room temperature. The tube was opened, two volumes of ether was added, and the mixture was transferred to a centrifuge tube, which was capped to exclude moisture. After being washed twice with dry ether by centrifugation, the yellow imidoester dihydrochloride was dried in high vacuum; it weighed 590 mg. The product was used in the next step without further purification.

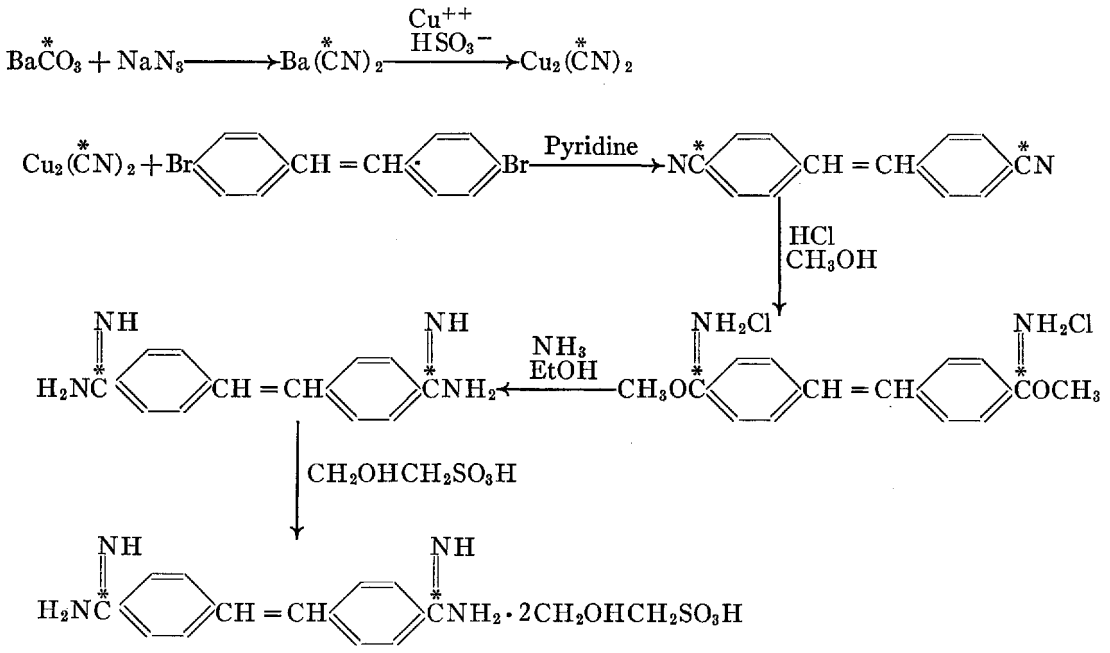

CHART 1

Stilbamidine diisethionate.—The imidoester was transferred to a 15 × 150 mm. Pyrex ignition tube, and the neck was constricted far enough below the mouth to avoid introduction of water from the flame. Into the tube was introduced 10 ml. of a 10 per cent solution of anhydrous ammonia in absolute ethanol, and the tube was sealed. After being heated 36 hours at 60°, the tube was opened and the contents collected by centrifugation. The white product weighed 410 mg. after being washed with two 5-ml. portions of dry ether and dried in high vacuum.

Purification was effected by conversion of the diamidine to the dihydrochloride, followed by reprecipitation of the free base. To accomplish this, the amidine, still contained in the centrifuge tube in which it was collected, was thoroughly moistened with 0.5 ml. of concentrated hydrochloric acid, and the mixture was diluted with 5 ml. of water. The mixture was warmed to 60° and centrifuged. The supernatant solution was pipetted into an Erlenmeyer flask, where it deposited yellow crystals of the hydrochloride on cooling. The residue in the centrifuge tube was extracted repeatedly with water and the supernatant solutions added to the flask. As each new portion was added to the contents of the flask, more hydrochloride separated, salted out by the relatively high concentration of hydrochloric acid in the solution. When this criterion showed that nothing more was being dissolved from the residue in the tube, the entire procedure was repeated. The second extraction produced a small additional crop of hydrochloride. The Stilbamidine dihydrochloride was then precipitated more completely from the solution by addition of concentrated hydrochloric acid to raise its concentration to about 2 M.

The pale yellow hydrochloride was collected on a filter, sucked as dry as possible, dissolved in 8 ml. of water, and the solution was treated with charcoal and filtered. From the nearly colorless filtrate, cooled in ice, the free base was precipitated with 6 M ammonia. The white product was centrifuged, washed once with a cold water, and dissolved without delay in a solution of 0.63 gm. of isethionic acid in 2 ml. of water. A slight residue was removed, and Stilbamidine diisethionate was precipitated by adding 20 volumes of acetone. The product was dissolved, reprecipitated, and dried. The pale yellow product weighed 278 mg. (34 per cent based on imidoester) and melted with decomposition and evolution of gas at 308°–312° (uncorr.). The over-all yield, based on cyanide, was 19 per cent.

Analysis: Calc. for $C_{20}H_{28}O_8N_4S_2$ C, 46.50; H, 5.46; N, 10.08; S, 12.4;

Found: C, 47.41; H, 5.30; N, 10.17; S, 13.0.

The specific activity was 5.0 μc. per milligram.

Animal Procedures

Bagg albino mice, 1–1½ years old, weighing between 26 and 32 gm., were given single intravenous injections into a tail vein of 0.4 mg. of labeled Stilbamidine diisethionate dissolved in 0.05 ml. of isotonic saline. The dose, in terms of radioactivity, was 4 to 5 million disintegrations per minute. Immediately after the injection, each mouse was placed in an individual metabolism cage designed to permit quantitative collection of the exhaled carbon dioxide in alkali as well as separate collection of urine and feces. The food and water supplies were arranged in such a way that they could not become contaminated by radioactive excreta. Urine and feces were collected from most of the mice at intervals for the first 4–6 days. Thereafter, animals were occasionally returned to the cages for collection periods of 24–96 hours. When not in a metabolism cage, each mouse was kept in a separate cage in order to prevent cross-contamination from another mouse.

At various intervals after injection animals were anesthetized with ether, and as much blood as possible was drawn from the ventricle or inferior vena cava. Residual blood was removed from the tissues by viviperfusion with isotonic saline, and the carcasses were dissected immediately. Bile was obtained from the gall bladder and marrow from a femur or, occasionally, the ilium. The intestines were washed to remove the contents. Tissues, feces, and blood were dried *in vacuo*, and in all cases radioactivity values are given in terms of dry weight of residue. Each tissue or an aliquot was burned in a combustion train to carbon dioxide, which was converted to barium carbonate and its radioactivity measured (4). Urine aliquots were allowed to dry in a porcelain boat and were then burned in the combustion train. It was found that the Van Slyke-Folch wet combustion method (14) failed to break down completely the Stilbamidine in a reasonable length of time. Most of the measurements were made with a Nucleometer, but some of the more weakly active specimens were assayed in an ionization chamber (10) attached to a vibrating reed electrometer and a Brown recorder.

RESULTS

Toxicity and radiation effects.—Of 31 mice injected, the first 5 died immediately. Thereafter, the injection was made slowly over a period of 2 minutes. When this technic was used no mice died from the effects of the drug. One died of an undetermined cause 7 weeks after injection. No other spontaneous deaths occurred. One animal is living and healthy 11 months after injection, and two others are healthy 7 months afterward. No instances of diarrhea, anemia, epilation, hemorrhage, or other evidence of radio-biological effects were observed.

Respiratory excretion.—Excretion of radioactive carbon dioxide was measured in six mice. Two of the animals excreted minute amounts of radioactive carbon dioxide during the first 24 hours, the total excretion amounting to less than 0.2 per

cent of the administered dose. In three others there was no detectable excretion of radioactivity in the 24 hours. Two of the three were observed for 4 days after injection and failed to excrete detectable amounts of isotope in the breath during this time. In order to rule out a delayed excretion of activity by this route a 24-hour collection was made of the breath of another mouse 2 weeks after injection, again with a negative result.

Excretion in the urine and feces.—The urinary excretion data are averaged from a total of twenty animals and the fecal data from eighteen animals. The points on the cumulative curve for urinary excretion (Chart 2) for the first 8 days are based on the following number of animals for each successive 24-hour period: 11, 9, 7, 8, 8, 3, 3, and 6. For fecal excretion: 10, 10, 7, 7, 6, 2, 2, and 4.

Large amounts of radioactivity were found in both urine and feces, the total fecal excretion during the first 8 days being more than 50 per cent greater than the urinary. The rate of urinary excretion reached a maximum during the first 24 hours (17 per cent of injected dose), while the fecal peak occurred during the second 24 hours (14 per cent of injected dose). Cumulative excretion curves are shown in Chart 2. By the end of the second day 50 per cent of the Stilbamidine had been excreted in the urine and feces combined, and 70 per cent was lost by the end of the fourth day. After the eighth day, excretion was less than 1 per cent of the administered dose per day, and by the 30th day about 97 per cent of the injected activity had been excreted. After 1½ months, the concentration of radioactivity in the excreta was too low to be measured by the Nucleometer.

Tissue distribution.—Table 1 shows the concentration of radioactivity in the tissues and Table 2 the total radioactivity. The highest concentration appears in the kidneys, followed in order by the liver, heart, lungs, and intestines, with smaller concentrations in the other tissues. The distribution pattern is established within the first 2 hours and does not change substantially thereafter, although the differentials tend to decrease.

We have no data on the rate of plasma clearance of Stilbamidine, but it is interesting that in as little as 2 hours after administration there was only a minute trace of radioactivity present in the red cells or plasma.

It may be seen that the specific activity in the marrow is relatively small, but a trace of activity is still present 6 months after administration.

The ovaries contained relatively little activity at any period, but there was a small amount of residual activity at the 2-month level. The 6-month mouse was the only male in the tissue distribution series. The testes at this time contained a trace of activity (0.01 per cent of the injected dose in the total testes). On the bottom of Table 2 there is a summary of the total tissue content at each period. When added to the amount excreted up to the time of autopsy, the total activity accounted for agrees quite well with the amount injected. The greatest discrepancy occurred in the 24-hour mouse. Here only 81 per cent was recovered. The carcass was not available for assay, having been eaten by a rat who had gained access to the building. It is known that

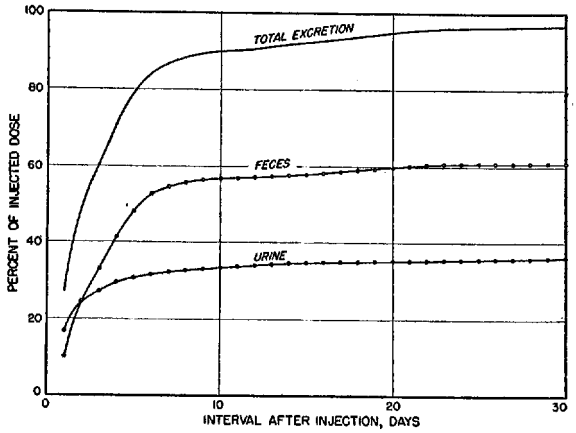

CHART 2.—Accumulated excretion

Excreta between 1 and 1½ months not collected; estimated at 0.03 per cent per day average. Feces collected from five mice at 1½ months were too weakly active to be assayed with the Nucleometer. Urine specimens from two mice at 1½ months contained zero and 0.3 per cent, respectively. Fecal specimens from four mice at 1½ months contained zero activity. Urine and feces from several mice thereafter at 15-day intervals to the 4-month period assayed zero.

the paw pads fluoresce soon after injection (8). This is undoubtedly due to contamination with urine and feces. Since large amounts of activity are in the urine and feces during the first 24 hours, it is quite likely that an appreciable amount of Stilbamidine was clinging to the paw pads of this mouse.

For the 4-day animal, the pelt included the site of injection and paw pads at the time of combustion, while in the other animals only an aliquot of pelt was combusted and the paw pads and tail were left with the carcass. A small aliquot of skin of the 4-day animal was combusted for comparison with total pelt, and it was found that the specific activity was only 0.0008, which is in the range of specific activity of the pelt aliquots of the 24-hour and 2-week animals. This is evidence that the relatively high pelt activity recorded in Tables 1 and 2 for the 4-day animal does not repre-

TABLE 1

SPECIFIC ACTIVITY AS PERCENTAGE OF INJECTED DOSE PER MILLIGRAM DRY TISSUE

	2 hr.	6 hr.	24 hr.	4 days	2 wk.	7 wk.	8 wk.	3 mo.	6 mo.
Kidneys	.128	.156	.156	.067	.022	.012	.016	.015	.001
Liver	.050	.096	.061	.026	.015	.002	.002	.001	.0004
Heart	.012	.012	.019	.016	.025	.008	.020	.007	0
Lungs	.005	.011	.006	.006	.007	.004	.002	.001	.0003
Gonads		.002	.0007	.0005	.001		.0004	0	.0003
Intestines	.004	.007	.005	.003	.0006		0	.0005	0
Intest. contents	.007	.006	.013	.009				0	
Bone	.0009	.0004	.0021	.0001	.0007	.0004	.0001	.00001	
Bone minus marrow		.0011	.0005		.0002	.0011	.00006	.0001	
Marrow		.0073	.0028		.0013		.0058	.0044	.0003
Bile		.06	.35		.003		.013	.003	.0003
Gall bladder		0	.042		.018		0	0	
Plasma	.0004	.0001	.0001	.0016				0	
RBC	.0002	.0002	.0001	.0007			0	0	
WBC								0	
Pancreas		.001	.002	.004	.001			0	
Spleen	.0009	.002	.002	.003	.0009	.003	.001	0	0
Lymph nodes		.0005	.004	.0007	0		0	0	0
Muscle		.001	.0012	.0016	.0012		.0014	.0006	.00001
Stomach		.004	.003	.003	.002		0	.0003	.00004
Eyes		0	0	.001	.001				
Brain		.0004	.0004	.0006	.0009			0	
Pelt			.0004	.003	.0002			0	0
Adrenals		.0042	.0094		.0052		.0048	.0014	.0009

TABLE 2

TOTAL ACTIVITY AS PERCENTAGE OF INJECTED DOSE

	2 hr.	6 hr.	24 hr.	4 days	2 wk.	7 wk.	8 wk.	3 mo.	6 mo.
Kidneys	12.2	15.9	17.5	5.97	2.55	.96	1.53	1.19	.17
Liver	20.6	46.4	31.7	8.74	6.50	1.06	.75	.40	.19
Heart	.36	.38	.61	.43	.79	.28	.51	.19	0
Lungs	.19	.55	.30	.20	.37	.18	.06	.04	.01
Gonads		.39	.30	.38	.23		.18	0	.01
Intestines	2.36	4.77	2.59	.66	.31		0	.03	0
Intest. contents	.864	1.29	1.58	.49				0	
Bone		1.48*	.68	3.45	.19*	1.15*	.71*	.17*	.02*
Gall bladder		0	.02		.01		0	0	
Plasma								0	
RBC							0	0	
WBC								0	
Pancreas		.24	.33	.13	.38			0	
Spleen	.04	.13	.11	.07	.05	.08	.02	0	0
Lymph nodes					0		0	0	0
Muscle		1.71*	1.95*	2.78	2.00*		2.26*	.98*	.01*
Stomach		.56	.32	.14	.17		0	.03	.004
Eyes		0	0	.01	.02				
Brain		.04	.03	.05	.10			0	
Pelt			.96*	6.80	.44*			0	0
Adrenals		.028	.071		.040		.014	.013	.003
Carcass or remnant	62.10	19.34			7.92				1.2
TOTAL:	98.71	93.21	59.05	30.30	22.07		6.03	3.04	1.62
Less estimated tissues (these tissues included in carcass)		3.19			2.63				
Total in tissues	98.71	90.02	59.05	30.30	19.44		6.03	3.04	1.62
Urine	2.88	10.05	13.30	29.46†	34.64†		36.96†	37.†	37.†
Feces	.04	1.37	8.42	39.88†	55.58†		60.80†	61.†	61.†
Total accounted for:	102	101	81	100	110		104	101	100

* Total muscle weight estimated.

† From the average excretion data of all the animals (Chart 2).

"0" Indicates activity too near background for significant measurement.

sent generalized high pelt localization but rather a concentrated local contamination, such as might be expected on the paws and tail.

DISCUSSION

Storage in the body.—Henry and Grindley, using the fluorimetric method of analysis, added Stilbamidine to whole sheep blood and observed that most of it disappeared from the plasma (9). They concluded that erythrocytes have a high adsorptive capacity for the drug, although its presence in them could not be directly demonstrated, and suggested that in the body it is stored mainly in the erythrocytes. Fulton and Goodwin repeated these experiments using a spectrophotometric method of analysis and were unable to confirm the earlier findings (5). They concluded that absorption of Stilbamidine by red cells does not occur to any appreciable extent. Table 2 shows that *in vivo* the erythrocytes contain a very small proportion of the total stored radioactivity, most of which is located in the liver and kidneys with modest amounts stored in intestine, total muscle, bone, and pelt. Hawking and Smiles had concluded earlier from a qualitative examination under ultraviolet light of the tissues of mice given the drug that most of it is located in liver, kidneys, intestine, paw pads, and injection site (8).

Nothing is known of the form in which Stilbamidine is stored. It may be pointed out, however, that Hawking and Smiles could observe no fluorescence of the kidneys 4 days after injection, although the concentration of radioactivity in these organs at this time is still greater than the initial liver concentration, which was sufficient to give a visible fluorescence. It is therefore likely that Stilbamidine is converted in one or more of the tissues of the body to a different chemical form.

Urinary excretion.—The urinary excretion of Stilbamidine administered subcutaneously to rats has been studied by Wien (15) and by Hampton (7). Using the fluorimetric method, Wien observed an excretion of 1.0 per cent, and Hampton found 1.7 per cent in the urine during the 24 hours following a single dose of 5 mg/kg. Both reported that, with the glyoxal method of analysis, values as much as 20 times higher were found. The glyoxal values and our own of 17 per cent are in fair agreement, but it is clear that the fluorimetric method demonstrates only a fraction of the total excretion of the drug and its metabolites. The explanation of the discrepancy between the glyoxal values and ours may lie in the finding of Wien, confirmed by Hampton, that with either method of analysis the percentage excretion in the 24 hours following a single dose decreases with increasing size of the dose. Our dosage was 20 mg/kg.

The rapidity of the drop in urinary excretion rate shown in Chart 2 is in agreement with the results of Hawking and Smiles (8), who were unable to detect any fluorescence 2 days after injection.

Fecal excretion.—The fecal excretion of Stilbamidine, which constitutes a major mode of elimination, has not been previously detected. Table 1 shows that the bile carries a relatively high concentration of radioactivity, a specimen taken from the gall bladder 24 hours after injection showing a concentration of 0.35 per cent of the dose per milligram of solids. Since the daily bile secretion of the mouse is about 2 ml., containing about 280 mg. of solids, it is clear that a large part—possibly all—of the radioactivity entering the feces is carried by the bile. Since Hawking and Smiles could detect no fluorescence in the bile of mice receiving Stilbamidine, it is likely that the drug has undergone chemical transformation.

SUMMARY

1. Stilbamidine diisethionate labeled with carbon 14 has been prepared and its distribution studied in mice.

2. After intravenous administration, 70 per cent is excreted within 4 days, the remainder being excreted much more slowly, reaching 97 per cent on the 30th day. Much of the excreted material has undergone chemical transformation. Excretion occurs via the urine and feces.

3. The highest concentration of radioactivity appears in the kidneys, liver, heart, lungs, and intestines. Traces of radioactivity are detectable in many tissues 6 months after injection.

4. Stilbamidine is not metabolized to carbon dioxide in the mouse.

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The Fixation of Urethan Carbon Atoms in Sperm and in Resting and Rapidly Dividing Cells*

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As a result of various studies on the mechanism of action of urethan (ethyl carbamate), considerable data are available which might be used to formulate theories worthy of investigation. It is difficult, however, on reviewing the literature, to determine which of the numerous cytologic, histopathologic, and biochemical responses observed with this compound are fundamental to the anticancer activity of urethan and which are only secondary manifestations related or unrelated to its chemotherapeutic activity.

Urethan appears to be a highly specific molecule with regard to anti-leukemic action. Changes in the ester group, mono- or disubstitution at the nitrogen atom, and substitution of sulfur for the carbonyl or ethoxy oxygen, all destroy or lessen the anti-leukemic action of urethan in mice with chloroleukemia 1394 (10). There is apparently no correlation between the anti-leukemic and the leukopenic action of carbamates (10, 11), but there is a correlation between the anti-leukemic and carcinogenic activity (7–10), and there is also a correlation between the leukopenic action observed in mice (11) and the inhibition of sea-urchin egg cleavage caused by substituted carbamates (2). These facts would seem to suggest that two basic mechanisms may be involved in therapy with ethyl carbamate, one having to do with cell division and the other with differentiation processes.

Over-all tracer studies in mice have shown that the carbonyl and methylene-carbon atoms of urethan are fixed in all tissues, but to no greater extent than can be accounted for by fixation of corresponding atoms from CO_2 and ethyl alcohol, the probable *in vivo* hydrolysis products of this compound.[1]

The present experiments were designed with the thought that studies on the fate of urethan in a controlled population of rapidly dividing cells and in cells with a large proportion of desoxyribonucleoprotein (sperm) might yield clues to the role of urethan in normal and neoplastic proliferation.

Briefly, we have investigated the accumulation of urethan in resting and dividing sea-urchin eggs and also the fixation of the carbonyl and methylene carbon atoms of urethan in eggs and in sperm. Control experiments have been carried out with the *in vivo* hydrolysis products of urethan: CO_2 (as $NaHC^{14}O_3$) and methylene-labeled ethyl alcohol.

EXPERIMENTAL

Fertilized and unfertilized eggs from the sea urchin, *Tripneustes esculentus*, supplied the dividing and resting cells. These eggs normally divide at about 70 minutes after fertilization. For studies on cells with a high proportion of desoxyribonucleoprotein, sperm from a large sea cucumber were employed. The temperature of the sea water in which the eggs and sperm were suspended ranged from 25° to 27° C., with no more than 0.4° variation during the course of a single experiment.

ACCUMULATION OF URETHAN BY EGGS

Experiment No. 1.—Eggs were exposed to 20 mg. of urethan per milliliter of egg suspension (in sea water), beginning 10 minutes after fertilization. Samples were then centrifuged, quickly washed in sea water, and recentrifuged. They were then fixed in 5 ml. of saturated $HgCl_2$. The exposure durations are given in minutes and seconds in Table 1. The eggs were analyzed for their urethan content, by the procedure of Boyland and Rhoden (1). From this concentration (column 4, Table 1) and the dilution with $HgCl_2$, the concentration in the eggs at the time of fixation

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† Stabilite Division, Food and Container Institute, Chicago, Illinois.

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[1] H. E. Skipper, L. L. Bennett, Jr., C. E. Bryan, L. White, Jr., M. Newton, and L. Simpson, unpublished data.

(column 5) was calculated. Eggs left in the urethan solution at the time of the experiment did not divide. Samples removed to sea water simultaneously with fixation of the other samples did divide, and those removed and placed in urethan-free sea water even after 1 hour of exposure formed gastrulae.

Experiment No. 2.—Fertilized and unfertilized eggs were exposed to 2 mg. of urethan per milliliter of egg suspension. Exposure of fertilized eggs began 10 minutes after fertilization. The eggs were

TABLE 1

ACCUMULATION OF URETHAN BY FERTILIZED SEA-URCHIN EGGS*
(Initial Concentration 20 mg/ml)

Sample no.	Exposure† period	Weight of eggs (mg.)	Analyzed urethan (mg/gm eggs)	Calculated conc. in eggs at fixation‡ (mg/gm)
1	2′44″	852	3.5	25.1
2	5′22″	988	3.5	21.8
3	7′59″	700	3.3	28.9
4	10′44″	842	3.4	24.6
5	19′28″	844	4.9	35.4
6	49′15″	914	2.1	14.7
7	80′15″	727	2.9	24.0

* Urethan added 10 minutes after fertilization.

† Duration of exposure in minutes and seconds between addition of urethan and washing.

‡ The calculated concentration represents: (conc. of urethan determined by analysis) × (total wt. of eggs + fixative) ÷ (wt. of eggs).

TABLE 2

ACCUMULATION OF URETHAN BY FERTILIZED AND UNFERTILIZED SEA-URCHIN EGGS*
(Initial Concentration 2 mg/ml)

Fertilized		Unfertilized	
Exposure	mg/gm eggs	Exposure	mg/gm eggs
1′18″	1.90	1′10″	1.18
2′28″	4.60	2′23″	1.50
4′26″	2.07	4′1″	1.60
21′22″	2.21	21′12″	1.36
53′40″	1.12	56′11″	1.94

* Urethan added 10 minutes after fertilization. Exposures measured from that instant to time of freezing in minutes and seconds.

centrifuged, the sea water decanted, and the eggs frozen and kept in this condition until analyzed for urethan content (Table 2). Fertilized eggs left in the urethan-containing sea water developed more slowly than the controls but eventually formed blastulae.

Tracer Experiments

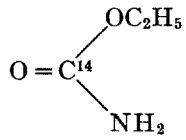
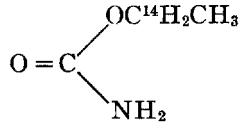
Urethan was labeled in the carbonyl position ($H_2NC^{14}OOC_2H_5$) or the methylene position ($H_2NCOOC^{14}H_2CH_3$). Labeled bicarbonate and methylene-labeled ethyl alcohol were used as controls. The activities of each, as well as concentrations of the solutions employed, are presented in Table 3. The labeled compounds used in this study were synthesized by procedures which will be reported elsewhere.[1] All samples were assayed for carbon 14 by a gas-phase procedure already described (12), and tabulated as specific activity—i.e., μc. of activity per mole of carbon in the eggs or sperm.

Fertilized and unfertilized eggs were exposed to the four labeled compounds, then were concentrated and quick-frozen in a dry-ice acetone bath and were kept frozen until they could be assayed for fixed activity (Tables 4 and 5).

The sperm were obtained by mechanically shredding the testes of the sea cucumber and filtering the sperm through gauze. Enough sea water was added to activate half the sperm (all sperm

TABLE 3

CONCENTRATIONS AND ACTIVITIES OF COMPOUNDS USED

Compound	Structure	Activity (μc/ml)	Concentration (mg/ml)
Carbonyl-labeled urethan		6	175
Labeled sodium bicarbonate	$NaHC^{14}O_3$	10	86
Methylene-labeled urethan		7	175
Methylene-labeled ethyl alcohol	$HOC^{14}H_2CH_3$	7	90

showed activity when dispersed in a large volume of sea water). To each 50 or 100 ml. of the sperm suspension, 0.2 ml. of one of the labeled compounds was added. The samples were quick-frozen at 30 minutes and kept frozen until analyzed (Table 6).

The values reported as *Free+fixed* urethan refer to specific activities of the frozen samples as such. The fixed activities were determined on aliquots subjected to repeated suspension in water (weak acid in the case of $NaHC^{14}O_3$) and vacuum drying (24 hours at 1 mm.) to remove the free compounds. Three such treatments were found to reduce the activity of exposed urchin eggs to a constant value, after which no additional activity was removed by this procedure. We have repeatedly shown that free urethan can be removed from biological specimens by vacuum distillation. In one experiment, an aliquot of each sperm sample was dialyzed against cold running water for 24 hours prior to activity assays (Table 6).

RESULTS

Within 2 minutes, fertilized eggs accumulated a higher concentration of urethan than was present in the surrounding medium. This occurred at a concentration which prevented division (20 mg/ml: Column 5, Table 1), or at a lower dose which merely retarded development (2 mg/ml: Column 2, Table 2).

Only a small proportion of the urethan which entered fertilized or unfertilized eggs (Table 4, *Free+fixed*) was not removed by vacuum distillation. The amount of urethan carbon which was fixed by the cells was no greater than the carbon fixed from alcohol or bicarbonate (*Fixed corrected*, Table 4). Fixation was about equal for carbonyl and methylene urethan carbons and bicarbonate, and the ratio was unaltered with longer or shorter exposure (Table 5). Fixation of ethanol carbon was higher than the other three but declined slightly with prolonged exposure.

In sea-cucumber sperm the ratio of fixation of urethan carbon to alcohol or bicarbonate carbon was higher than in the eggs, and in most instances the sperm fixed more of the urethan carbon than carbon from alcohol or bicarbonate. This was true when fixation was tested by vacuum distillation (Table 6, Exp. No. 1) or by dialysis (Exp. No. 2). Extraction of desoxyribonucleic acid (DNA) from the sperm resulted in loss of fixed carbon, with the DNA retaining more of the ethanol or bicarbonate carbons than urethan carbons.

DISCUSSION

It must be emphasized at the onset of this discussion that the experiments reported herein are exploratory in nature and were designed to obtain qualitative evidence of the relationship between the reaction of parts of the urethan molecule with the whole resting and dividing cell and with a living substance (sperm) which is predominantly

TABLE 4

FIXATION OF CARBON 14 FROM LABELED COMPOUNDS BY FERTILIZED AND UNFERTILIZED SEA-URCHIN EGGS

	Unfertilized Eggs*				Fertilized Eggs†			
	Activity added	Specific activity‡			Activity added	Specific activity‡		
Compound	(μc/50 ml)	Free+fixed	Fixed	Fixed corrected	(μc/50 ml)	Free+fixed	Fixed	Fixed corrected
Carbonyl-labeled urethan	0.6	2.67	0.014	0.023	0.24	0.68	0.007	0.012
Sodium bicarbonate	1.0		0.035	0.035	0.40	0.17	0.010	0.010
Methylene-labeled urethan	0.7	2.84	0.010	0.014	0.28	1.09	0.010	0.014
Methylene-labeled ethyl alcohol	0.7	1.66	0.090	0.129	0.28	0.90	0.050	0.072

* Exposed for 29 minutes.

† Exposed for 35 minutes beginning 31 minutes after fertilization.

‡ Specific activities in μc/mole of egg carbon. Corrected for the differences in total activity added to eggs.

TABLE 5

THE SPECIFIC ACTIVITIES OF FERTILIZED SEA-URCHIN EGGS AT VARIOUS PERIODS AFTER EXPOSURE TO CERTAIN C^{14}-LABELED COMPOUNDS*

	Activity added	Amount added	Specific activity fixed† exposure period (minutes)				Fixed corrected‡
Compound	(μc/50 ml)	(mg/50 ml)	(11)	(30)	(48)	(78)	(30 min.)
Carbonyl-labeled urethan	0.6	17.5	0.03	0.04	0.04	0.07	0.07
Sodium bicarbonate	1.0	8.6	0.03	0.03	0.05	0.09	0.03
Methylene-labeled urethan	0.7	17.5	0.02	0.05	0.03	0.05	0.07
Methylene-labeled ethyl alcohol	0.7	9.0	0.15	0.14	0.10	0.07	0.20

* Exposure begun at 6½ minutes after fertilization.

† Specific activity in μc/mole of egg carbon.

‡ Corrected for the differences in total activity added to eggs.

TABLE 6

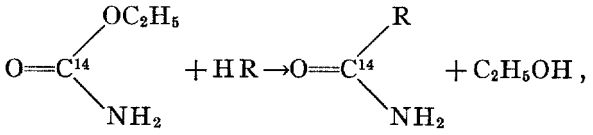
FIXATION OF CARBON 14 FROM LABELED COMPOUNDS BY ACTIVATED SEA CUCUMBER SPERM*

	Experiment No. 1			Experiment No. 2			
			Specific activities			Specific activities	
Compound	Activity added (μc/50 ml)	Amount added (mg/50 ml)	vacuum-treated	Activity added (μc/50 ml)	Amount added (mg/50 ml)	Vacuum-treated	Dialyzed
Carbonyl-labeled urethan	0.6	17.5	0.73	1.2	35.0	0.18	0.022
Sodium bicarbonate	1.0	8.6	0.08	2.0	17.3	0.02	0.008
Methylene-labeled urethan	0.7	17.5	0.46	1.4	35.0	0.24	0.099
Methylene-labeled ethyl alcohol	0.7	9.0	0.14	1.4	18.1	0.21	0.019

* Exposure 30 minutes. Specific activity in μc/mole of sperm carbon, corrected for differences in total activity added.

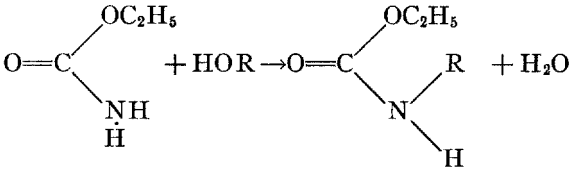
genetically active material, i.e., desoxyribonucleoprotein. Despite fluctuations in the data which cannot be explained at this early stage, definite trends are discernible which shed light on the mechanism of action of urethan.

In the tracer experiments, the fixed atoms are presumably, although not necessarily, combined chemically with some cell component. If the carbonyl-carbon atom of urethan reacted extensively with active groups of amino acids important in cell division as suggested by Dustin (4),



it is possible that our experiments would have demonstrated a high degree of carbonyl-carbon fixation as compared to the methylene-carbon atoms.

If urethan *per se* were adsorbed to cell fractions or reacted through the amide group as follows:



then one might expect to find both the carbonyl and the methylene-carbon atoms fixed to a greater extent than corresponding atoms from *in vivo* hydrolysis products of this molecule, CO_2, and ethyl alcohol.

Our present results would seem to indicate that unfertilized (resting) and fertilized (dividing) sea-urchin eggs fix both the carbonyl and the methylene atoms of urethan to a lesser extent than the methylene carbon of ethyl alcohol. The labeled carbon from $NaHC^{14}O_3$ was fixed (probably by entering the normal metabolic pathways) at a rate somewhat similar to that of the above-mentioned urethan carbon atoms.

The fixed urethan represents only a small portion of the urethan accumulated in the cell. The present observations suggest that the removable urethan is the fraction responsible for inhibition of mitosis, since on washing the eggs quickly recover. It possibly follows that fixing of urethan carbon in these eggs might be accounted for along paths having little to do with cleavage. At least, ethanol is eliminated as the route by which urethan affects cell division. For, although ethanol penetrates in comparable amounts (cf. *Free+fixed*, Table 4) and contributes more fixed activity in the eggs, 10 times as much ethanol as urethan is required to retard division of Tripneustes eggs (Cornman, unpublished data).

At this point in the argument, however, it is well to recall that the doses used in the tracer experiments were a tenth as high as the levels needed to retard cleavage. One may suspect that the fate of urethan in eggs will change as the physiology of the egg is altered by the narcotic. Even within the division-inhibiting range, the dose-effect relationships of sea-urchin eggs are different at low and high concentrations (5). Accordingly, it may prove worth-while to investigate the effect of urethan itself on the fixation of labeled urethan.

Klotz *et al.* (6), in a mass law analysis of the binding ability of bovine albumin, have shown that urethan is capable of displacing anions from a protein complex. This reaction was observed within a period of minutes, insofar as displacement effects were involved, and is additional evidence of the fixation of at least a part of the urethan molecule to protein.

Perhaps the most interesting portion of this study is the section of the results presented in Table 6 which indicate that sperm fix more urethan carbon in proportion to bicarbonate or ethanol carbon than do eggs. In this relationship the urethan methylene carbon is the more consistent, being fixed in greater amounts than the ethanol methylene carbons by sperm, and in lesser amounts than the ethanol by eggs. This same relationship was true in sperm dialyzed for 24 hours, despite the greater loss of radioactivity which may well represent the removal of dialyzable urethan fixation products. This preferential fixation of urethan carbon in cells with a high proportion of desoxyribonucleoprotein, when considered in the light of mutagenic, carcinogenic, and anti-cancer activity of this compound, is, to say the least, suggestive. Cowen (3) has reported an inhibition of urethan carcinogenesis by pentose nucleotides.

SUMMARY

Fertilized eggs of *Tripneustes esculentus*, when exposed to concentrations of urethan which reversibly inhibited division or to lower concentrations which retarded division, rapidly accumulated urethan in concentrations equal to or exceeding the surrounding concentration. Unfertilized eggs accumulated urethan more slowly and did not exceed the surrounding concentration.

Most of the urethan accumulated in the eggs was in a free or loosely bound state. Cells inhibited by this free urethan began to divide again, once the urethan was removed by washing.

Using tracer technics, it was shown that fertilized and unfertilized eggs fixed only a very

small portion of the carbonyl and methylene-carbon atoms from their accumulated urethan; no more than might be accounted for by fixation of carbon from urethan hydrolysis products, CO_2 and ethyl alcohol.

Sea-cucumber sperm fixed more of the urethan carbon in proportion to carbon from labeled bicarbonate or ethanol than did eggs.

It is suggested that urethan is accumulated within the dividing cell and exerts its effect on mitosis while free or loosely bound within the cell. However, the specific fixation of carbon from two groups in the urethan molecule to sperm (high in desoxyribonucleoprotein) suggests that this compound combines with nuclear material. This affinity for nucleoproteins may be related to the well-known mutagenic, carcinogenic, and anti-cancer activity of urethan, since such processes are often considered to be associated with nucleoprotein metabolism.

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The Influence of Dietary Fat and Riboflavin on the Formation of Spontaneous Hepatomas in the Mouse*†

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These experiments are part of an investigation on the influence of nutrition on the formation of spontaneous hepatomas in mice. Attention is being given to those dietary factors known to affect the formation of other tumors in the mouse or the induction of hepatic tumors in rats to whom carcinogenic azo dyes have been administered. It has been shown that the formation of the spontaneous hepatoma in C3H male mice is significantly inhibited by restriction of caloric intake (14), by a diet low in protein (13), and probably by a "brown rice" diet (11). This communication is concerned with the effects of fat-enrichment of the diet and of rations low in riboflavin.

METHODS

The mice employed were inbred strain C3H males raised in our laboratories. Spontaneous benign hepatomas are first observed when the animals on common laboratory rations are about 10 months old; generally, about 40 per cent of the mice develop these tumors by 14 months of age.

The mice of each experiment were born within a span of 1–2 weeks, and approximately 50 animals were incorporated by random distribution into each group. They were fed Purina Laboratory Chow checkers until the experimental rations were instituted. The procedures for preparations of diets, housing of animals, and periodic weighing and inspection were similar to those previously described (13).

The individual mice maintained comparatively constant body weights between the time they were 10 months old and the termination of the experiment. Inasmuch as this is also the interval during which hepatomas arise, the data on body weights are summarized for this period only; the tables give the range of individual values within each group and the means of the values. At the conclusion of the experiment the mice were examined for grossly visible hepatomas.

EXPERIMENTS

Experiment 1.—Two groups of 50 3-month-old mice were employed. The mice of group n 40 were fed daily a "natural food" diet, consisting of 1.6 gm. Purina Fox Chow meal, 0.8 gm. skimmed milk powder, and 1.6 gm. cornstarch. The mice of group n 41 were fed the same ration, except that 0.56 gm. of fat (partially hydrogenated cottonseed oil) replaced an equicaloric amount of the cornstarch—1.4 gm. The diet for group n 40 contained 2 per cent fat, that for group n 41, 20 per cent fat. The mice of both groups ate all their food and grew equally well, increasing from a mean initial body weight of 28 gm. to approximately 39 gm. They maintained this latter weight from 10 to 13½ months of age, at which time the experiment was concluded.

In this study the fat-enriched diet did not accelerate the formation of hepatomas, the incidences in the two groups being virtually identical (Table 1). Neither was there any difference in the proportions of mice with more than one hepatoma, nor in the mean diameters of the hepatomas.

TABLE 1

EFFECT OF THE PROPORTION OF DIETARY FAT ON THE FORMATION OF SPONTANEOUS HEPATOMAS
(Experiment 1)

Group	Dietary fat* Per cent	No. of mice†	Body weight level‡ (gm.) Range	Mean	Mice with hepatomas No.	Per cent
n 40	2	50	35–43	39	22	44
n 41	20	47	33–45	39	22	47

* See text for description of diets. The mice of each group consumed 14 Calories daily.

† Number of mice sacrificed at 13½ months of age.

‡ Body weight levels of individual animals from the time they were 10 months of age until end of experiment.

Experiment 2.—Three groups of 4½-month-old

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mice were placed on the diets of semi-purified known components shown in Table 2. The ration given the control group BA 1 contained 2.3 per cent fat and furnished 16 μg. of riboflavin daily for each mouse; that for the high fat group BA 2 contained the same amounts of protein, vitamins, and minerals as the diet for group BA 1, but the fat content was raised to 21 per cent by equicaloric substitution for cornstarch; the diet fed the low riboflavin group BA 3 was identical with that for group BA 1, except that it contained only 2.0 μg.

TABLE 2

COMPOSITION OF THE DAILY RATIONS FED TO MICE OF EXPERIMENT 2

	BA 1	BA 2	BA 3
Kremax,* gm.	0.08	0.60	0.08
Cornstarch, gm.	2.55	1.25	2.55
Casein,† gm.	0.72	0.72	0.72
Gelatin,‡ gm.	0.08	0.08	0.08
Salts,§ gm.	0.14	0.14	0.14
Vitab,‖ gm.	0.03	0.03	0.03
A, D, E concentrate,# ml.	0.005	0.005	0.005
Riboflavin,** μg.	16	16	2.0

* Partially hydrogenated cottonseed oil (Armour & Co., Chicago).

† Labco "Vitamin Free" Casein (Borden Co., New York).

‡ Edible gelatin (Ucopco 14X, Wilson & Co., Chicago). It was dissolved in water and added to the diet, serving as a binder.

§ Wesson modification of Osborne-Mendel Salt Mixture (15).

‖ Rice bran concentrate (Nopco Chemical Co., Harrison, N.J.); contains approximately 8 μg. of riboflavin per gram.

This concentrate supplied 20 U.S.P. units of vitamin A, 2 U.S.P. units of D, and 0.8 mg. of E in 0.005 ml. of cottonseed oil.

** Total riboflavin in the daily ration including that present in the Vitab. In addition, each mouse received the following B-Vitamins: thiamine HCl, 56 μg.; pyridoxine HCl, 48 μg.; niacin, 170 μg.; Ca pantothenate, 120 μg.; choline Cl, 4 mg.

of riboflavin. The mean body weights of the mice of groups BA 1 and BA 2 increased from an initial value of 31 gm. to 39 gm. at 8 months of age. During the same interval the mice of group BA 3 (low riboflavin) maintained an average body weight of 32–34 gm. and consumed about 5 per cent less food than the mice of the other two groups. In the ninth and tenth months a number of the mice of each group had diarrhea and lost weight; a few died. This disorder subsided, and the body weights were stabilized during the remainder of the experiment (Table 3).

When the mice were 6 months of age, four animals from each group were sacrificed to determine the riboflavin content of the livers. This procedure was repeated at 10 months and gave essentially the same results. The mean concentrations of riboflavin were 31.2, 31.7, and 28.6 μg. per gram in groups BA 1, BA 2, and BA 3, and the total amounts per liver were 52.9, 58.0, and 38.4, respectively.

The remaining mice were examined for the presence of hepatomas when they were 13 months old; the data are given in Table 3. In comparison with the control group BA 1, the mice fed the high fat ration, group BA 2, had a higher incidence of hepatomas, whereas those fed the low riboflavin diet, group BA 3, had a lower incidence. However, neither difference is of statistically significant magnitude.

Experiment 3.—In view of the difficulties encountered and the lack of decisive results in the preceding study, another experiment was performed. It differed from the former in the following respects: (*a*) to ensure equicaloric food consumption by the three experimental groups, the rations were fed at a slightly restricted level (10.2 Calories daily); (*b*) the difference in riboflavin content between the control and low riboflavin diets was increased: each mouse of groups BF 1 (control) and BF 2 (high fat) received 30 μg. of riboflavin daily, those of BF 3 (low riboflavin) only 1.8 μg.; (*c*) the daily supplement of B-vitamins was increased for each mouse to 60 μg. thiamine HCl, 60 μg. pyridoxine HCl, 180 μg. Ca pantothenate, 300 μg. niacin, 12 μg. folic acid, 0.3 μg. biotin, 600 μg. *p*-aminobenzoic acid, 3 mg. inositol, 15 mg. choline Cl; (*d*) the mice were 6 months old when the experimental diets were initiated.

At the beginning of the experiment the mean body weights of the mice of the three groups were 36 gm. Groups BF 1 and BF 2 nearly maintained this weight level, as shown in Table 4. The mean body weight of group BF 3, however, declined to 31 gm. within 6 weeks, increased to 34 gm. in the following 6 weeks, and then declined to 31 gm.; this level was maintained during the final 4 months of the experiment.

The results (Table 4) are in essential agreement with those of Experiment 2. Among the mice fed the fat-enriched ration (group BF 2) there was a somewhat higher incidence of hepatomas than among those fed the low fat ration (group BF 1), and the mice fed the low riboflavin ration (group BF 3) had a slightly lower incidence of hepatomas. There was no noteworthy difference among the groups in the proportion of mice with multiple hepatomas or in the size of the tumors.

DISCUSSION

Increasing the fat content of the diet from about 2 per cent to approximately 20 per cent may result in a small enhancement in the rate of formation of the spontaneous hepatoma of the mouse. While no augmenting effect was observed in Experiment 1, the hepatoma incidence rose from approximately 37 per cent to 53 per cent in Experiments 2 and 3. Moreover, in three studies relating to other types of tumors in the C3H mouse (in which diets similar to those of Experiment 1 were utilized), in-

cidental observations suggested that fat enrichment of the ration accelerates the rate of formation of spontaneous hepatomas.

With regard to other tumors of the mouse, an increase in the proportion of dietary fat from 2 to between 10 and 30 per cent markedly enhances the formation of the spontaneous mammary carcinoma, has a small but consistent augmenting effect on the formation of the induced skin tumor, but has little or no influence on the formation of the spontaneous lung adenoma or the induced sarcoma (1, 12). It has been reported that the induction of hepatic tumors in the rat by *p*-dimethylaminoazobenzene is accelerated by diets with moderate or high proportions of fat as compared to diets containing little fat (7, 4). However, this effect has not been found consistently (5, 10) and, furthermore, is dependent upon the type of fat (4).

Low dietary riboflavin resulted in an inhibition of the rate of formation of hepatomas; however, the effect can be principally ascribed to the lower body weight of the mice. The riboflavin deficiency was not of drastic proportions, causing only a 10 per cent decrease in the mean body weight; this reduction in body weight, even without a reduction in dietary riboflavin, can account for the observed decrease in hepatoma incidence (14).

The formation of spontaneous mammary carcinoma of the mouse was retarded by a riboflavin-deficient diet (6), but it is our opinion that the inhibition was due to a 30 per cent decrease in caloric intake and body weight of the animals. A ration low in both riboflavin and thiamine had no noteworthy influence on the formation of carcinogen-induced skin tumors (2). The incidence of hepatic tumors in rats administered *p*-dimethylaminoazobenzene is strikingly increased by a diet low in riboflavin but otherwise adequate (8); the enhancement varies considerably with the compound employed to induce the tumors (3). It is also claimed that high dietary riboflavin inhibits the formation of the hepatic tumors which are induced in some strains of rats by choline-deficient rations (9).

The available evidence indicates that low riboflavin diets have no specific effect upon the rate of formation of the spontaneous hepatoma, the spontaneous mammary carcinoma, and the carcinogen-induced skin tumor of the mouse. On the other hand, the porportion of dietary riboflavin strikingly influences the formation of hepatic tumors induced in rats by certain azo dyes.

SUMMARY

Fat enrichment of the diet from about 2 per cent to nearly 20 per cent appeared to enhance the rate of formation of the spontaneous hepatoma of the

TABLE 3

EFFECT OF THE PROPORTION OF DIETARY FAT OR RIBOFLAVIN ON THE FORMATION OF SPONTANEOUS HEPATOMAS
(Experiment 2)

	Diets*			Body weight level‡ (gm.)		Mice with hepatomas	
Group	Fat (per cent)	Riboflavin (μg. per day)	No. of mice†	Range	Mean	No.	Per cent
BA 1	2.3	16	44	29–41	34	17	39
BA 2	21.4	16	43	25–40	34	24	56
BA 3	2.3	2.0	48	26–36	31	9	19

* Diets fed *ad libitum;* see Table 2 for description. Mean daily caloric intakes for groups BA 1, BA 2, and BA 3 were 12.3, 12.2, and 11.8 Calories, respectively.

† Number of mice sacrificed at 13 months of age.

‡ Body weight levels of individual animals from the time they were 10 months of age until end of experiment.

TABLE 4

EFFECT OF THE PROPORTION OF DIETARY FAT OR RIBOFLAVIN ON THE FORMATION OF SPONTANEOUS HEPATOMAS
(Experiment 3)

	Diets*			Body weight level‡ (gm.)		Mice with hepatomas	
Group	Fat (per cent)	Riboflavin, (μg. per day)	No. of mice†	Range	Mean	No.	Per cent
BF 1	2.2	30	50	26–39	34	18	36
BF 2	20.4	30	47	30–41	35	24	51
BF 3	2.2	1.8	52	26–35	31	13	25

* Diets fed at equicaloric levels—10.2 Calories daily. See text for description.

† Number of mice sacrificed at 14 months of age.

‡ Body weight levels of individual animals from the time they were 10 months of age until end of experiment.

C3H mouse. Reduced dietary riboflavin, on the other hand, had no direct influence on the incidence of such hepatomas. These findings are discussed in relation to the effects of the two dietary modifications on the genesis of other tumors of the mouse and the azo dye-induced liver tumor of the rat.

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Further Evidence on the Mode of Action of 8-Azaguanine (Guanazolo) in Tumor Inhibition*

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One of the most interesting of the metabolic inhibitors is the triazolopyrimidine analog of guanine, 5-amino-7-hydroxy-1*H*-*v*-triazolo (*d*) pyrimidine. While this compound was found to be but moderately inhibitory to certain bacteria where no purine requirement existed (14), it is extremely inhibitory to the animal microörganism, Tetrahymena (6), which requires pre-formed guanine in its diet (7, 8). This guanine analog (guanazolo [9] or 8-azaguanine [18]) has an inhibition index of 0.02 for half maximum inhibition, and the inhibition is completely released by guanine (6). The above inhibition index indicates that, in order to obtain half maximum growth of Tetrahymena, the guanine concentration in the medium must exceed that of the 8-azaguanine by 50-fold.

Various considerations led to the hypothesis that this particular analog was being metabolized in place of guanine in Tetrahymena (6), and the suggestion was made that it might prove to be a powerful chemotherapeutic agent against any invading organism or abnormal cell which was able to metabolize guanine. Inasmuch as there was evidence that dietary guanine was not incorporated into the nucleoproteins of rats (2, 13) and that dietary adenine was so incorporated (2), the possibility existed that the guanine analog, 8-azaguanine, would prove to be relatively nontoxic to mammals. This was found to be the case with mice (9), and, moreover, remarkable carcinostatic powers toward three types of mouse neoplasms were discovered. The inhibitory action of 8-azaguanine toward certain types of mouse tumors was confirmed by Law (10), Gellhorn *et al.* (4), and Sugiura *et al.* (18), and in addition it was found that the Brown-Pearce undifferented squamous-cell carcinoma of the rabbit was also inhibited (4). It has also become evident that 8-azaguanine is entirely inactive against a large number of tumors in mice and rats (3, 4, 17, 18).

The hypothesis was advanced by Kidder *et al.* (9) that a biochemical difference existed between those neoplasms responsive to the inhibitory effects of 8-azaguanine and the host tissue. This qualitative difference was thought to be due to the ability of the tumor cells to incorporate pre-formed guanine (and hence 8-azaguanine) in contrast to the host nontumor cells. If the inhibitory effects on certain tumor cells by 8-azaguanine are indeed due to a changed biochemical pattern of metabolism, it might be explained either by the gain by the tumor cells of the ability to incorporate guanine or by the loss by the tumor cells of the ability to synthesize guanine—as is the case in Tetrahymena.

The present report will offer further evidence for the postulated qualitative biochemical difference between normal and certain neoplastic tissues.

MATERIALS AND METHODS

The tumors used in the present study were as follows: mammary adenocarcinoma 755[1] in C57 black line 6 mice; Sarcoma 180[2] in C3H or Swiss mice; Sarcoma 37[2] in C57 black mice; S 91[2] in dba line 1 mice; 6C3HED[2] (lymphosarcoma) in C3H mice; Lymphoma 2[3] (lymphoid leukemia) in hybrid A/C3H or A/Street mice; P1534[2] (lymphoid leukemia) in dba mice; spontaneous mammary adenocarcinoma[2] in Swiss mice. The bulk of the work to be reported here was done with adenocarcinoma 755. This tumor has several advantages

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[1] Obtained through the kindness of Dr. Alfred Gellhorn of the College of Physicians and Surgeons, Columbia University.

[2] Obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine.

[3] Obtained through the kindness of Dr. Lloyd W. Law, National Cancer Institute.

over E 0771 for use in metabolic studies. Its response to 8-azaguanine is more pronounced (4), and, because of its somewhat slower growth rate, the incidence of necrosis and sloughing is less, affording greater accuracy and reproducibility for quantitative measurements.

Due to the poor breeding capacities of strain A mice, experiments with Lymphoma 2 were carried out in F_1 hybrids of strain A crossed with C3H or Street. The Lymphoma 2 tumors used in transplanting all experimental series were carried in pure-bred strain A animals.

The technics of tumor inoculation were essentially the same as those used in other laboratories. Small fragments of the solid tumors were transplanted by trocar subcutaneously into the right axillary region of the experimental animals. The various leukemias were transplanted by means of intraperitoneal injections of saline cell suspensions of tumor masses dissected aseptically from donor animals (10). Variations in the number of leukemic cells in the suspensions accounted for the variation in survival times between experiments. Within any one experimental series the number of cells inoculated per animal was relatively constant.

The course of development of solid tumors was followed by caliper measurements. In experiments where more accurate quantitative data were necessary, the wet weight of freshly dissected tumors was determined (4). Survival time was employed as the significant end-point in the evaluation of experimental leukemias. Every animal was routinely examined at autopsy, as a precaution against the inclusion of "non-takes" in the data.

In preliminary experiments with tumor 755, it was noted that a small percentage of the untreated tumors grew much more slowly than did the average. It seemed desirable to eliminate these, if possible, from metabolic studies where optimum tumor behavior was demanded. In the following experiments, therefore, about 20 per cent more animals were regularly inoculated than were needed. Treatments were not instituted until 11 or 12 days after inoculation. At that time the experimental groupings were formed, the animals being distributed among the cages as equally as possible with regard to tumor size. The smallest 20 per cent were not used.

Water solutions of the triazolopyrimidines were used. The compounds were dissolved with the aid of dilute NaOH and finally adjusted to pH 7.8–8.0 with HCl. Intraperitoneal injections of 1-ml. volumes of the desired concentrations were employed. In these studies the total daily dose was given in a single injection (with one exception). Gellhorn *et al.* (4) found that divided doses of 8-azaguanine enhanced the response of tumor 755. We have confirmed this observation, but the requirements of these experiments did not demand the greatest possible response but, rather, comparisons between experimental groups. In the reversal experiments guanine was either injected subcutaneously in 0.2-ml. amounts as a suspension in peanut oil or fed after thorough mixing with pulverized Purina Chow pellets.

All animals were kept in Keystone plastic cages, ten per cage, with wood-shaving bedding, and food and water were offered *ad libitum*, except where guanine was added to the diet.

RESULTS

Distribution.—Sugiura *et al.* (18) investigated the effects of the administration of 8-azaguanine on a large number of experimental tumors of mice and rats. They report that the only tumor which was significantly inhibited was E 0771. They were unable to confirm our earlier findings (9) on the spontaneous adenocarcinoma of C3H mice. To extend the types of tumors investigated with this compound, we have studied the responses of the eight mouse tumor types listed in "Materials and Methods." We have confirmed the previous findings on tumor 755 (4), Sarcoma 180 (3, 4, 17, 18), 6C3HED (4) and Lymphoma 2 (9, 10). One of the most striking results of advanced lymphoid leukemia in Lymphoma 2-bearing animals is the enlarged and thickened spleen. When one compares the size (wet weight) of the spleens of untreated leukemic animals with those of the animals receiving 8-azaguanine, it is quite apparent that this compound is an active inhibitor, corroborating the evidence obtained on survival. The data in Table 1 demonstrate this point. We were unable to confirm the negative response of the spontaneous mammary adenocarcinoma in Swiss mice reported by Sugiura *et al.* (18). It has been our experience that this tumor is significantly inhibited by the administration of 8-azaguanine. The results of one of these experiments is shown in Chart 1.

TABLE 1

EFFECT OF 8-AZAGUANINE ON SPLEEN SIZE IN MICE WITH LYMPHOMA 2

	No. animals	Wet weight of spleens (Av. in mg.)
Normal controls (nonleukemic)	10	144
Leukemic controls (no treatment) 12 days after inoculation	10	1,298
Treated (8-azaguanine 65 mg/kg/day for 10 days) 12 days after inoculation	10	98

We found that the administration of 8-azaguanine to mice bearing Sarcoma 37 or Sarcoma 91

had an entirely negative effect. These two types, therefore, resemble Sarcoma 180. The lymphoid leukemia P1534 was inhibited by 8-azaguanine, as judged by survival time.

A summary of the results of the present investigation, together with those from the literature, is given in Table 2.

Dose response tests.—During the course of these investigations a large number of experiments have been performed to determine the relationship between the dosage of 8-azaguanine and the degree of tumor inhibition. The majority of these experiments was conducted on tumor 755, although in less complete series Lymphoma 2 and leukemia P1534 were used. In tumor 755 the amount of inhibition is directly proportional to the dosage of 8-azaguanine, up to about 50 mg/kg/day. The curve becomes asymptotic after this level and flattens out above 75 mg/kg/day. Chart 2 shows the dose response of tumor 755 to 8-azaguanine,

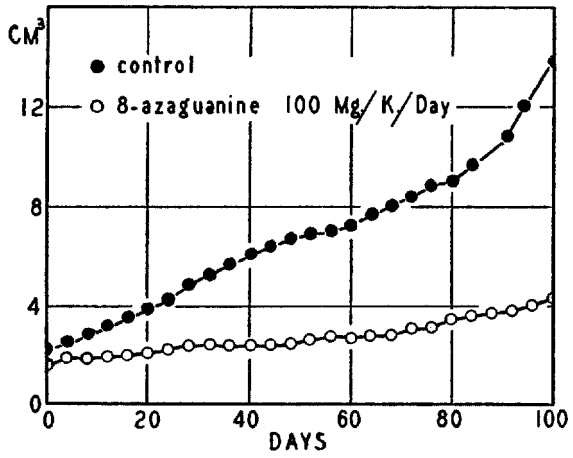

CHART 1.—Response of adenocarcinoma (spontaneous) of Swiss mice to 8-azaguanine. In this case treatments were given twice daily. There were eight animals in the control series and thirteen in the treated series.

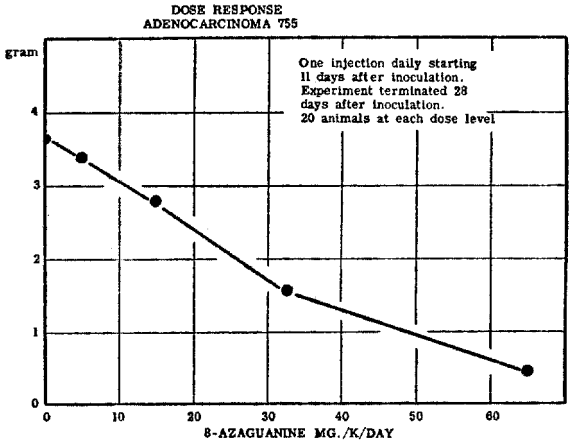

CHART 2.—Dose response curve to 8-azaguanine in Adenocarcinoma 755. Treatment in single daily doses. Figures on the ordinate represent average tumor weight in grams.

TABLE 2

SUMMARY OF EFFECTS OF 8-AZAGUANINE ON EXPERIMENTAL TUMORS

Tumor designation	Host	Type of tumor	Inhibition	Reference
E 0771	Mouse, C57 black	Mammary adenocarcinoma	+	(4, 9, 18)
755	" " "	" "	+	(4), present report
RC	" dba	" "	+	(4)
C3HBA	" C3H	Spontaneous mammary adenocarcinoma	+	(9)
C3HBA	" "	" " "	−	(18)
—	" Swiss	" " "	+	present report
—	" "	" " "	−	(18)
S 180	" Paris R III, C3H, Swiss, C57 black	Fibrosarcoma	−	(4, 17, 18), present report
S 37	Mouse, C57 black	Sarcoma	−	present report
T 241	" " "	"	−	(18)
Wagner	" AK	"	−	(18)
Harding-Passey	" "	Melanoma	−	(18)
S 91	" dba line 1	Malignant melanoma	−	present report
6C3HED	" C3H	Lymphosarcoma	−	(4), present report
Patterson	" AK	"	−	(18)
Lymphoma 1	" A	"	−	(10)
Lymphoma 2	" A, A/C3H, A/Street	Lymphoid leukemia	+	(9, 10), present report
P1534	" dba	" "	+	present report
L1210	" "	" "	+	(10)
AK4	" AK	Leukemia	−	(3)
9417	" "	"	−	(4)
R 39	Rat, Sherman	Sarcoma	−	(18)
August	" "	Carcinoma	+	(16)
Walker 256	" "	Carcino-sarcoma	−	(18)
Flexner-Jobling	" "	Carcinoma	−	(18)
Brown-Pearce	Rabbit	Undifferentiated squamous-cell carcinoma	+	(4, 15)
—	Rumex, Nicotiana	Virus wound tumor (plant)	+	(12)

and it will readily be seen that this curve resembles, to a marked degree, the typical dose response curves obtained with the use of inhibitors in microbiological studies.

There is considerable spread in the survival time of the treated leukemias. Averages of survival time, when plotted against dosage, produce smooth inhibition curves. Averages of survival

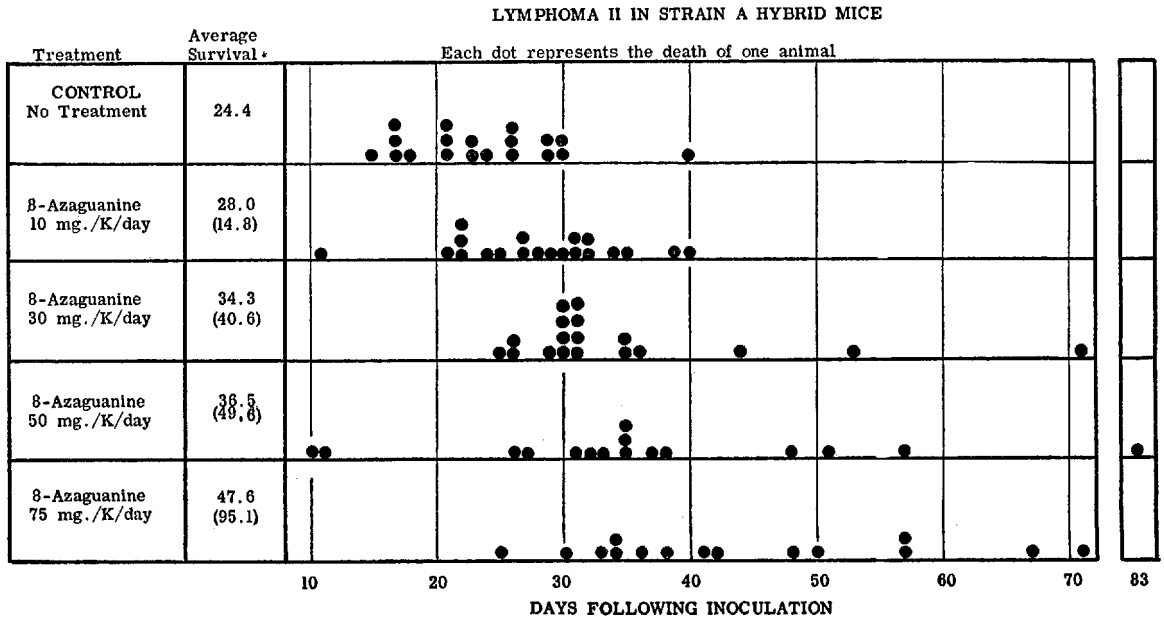

CHART 3.—Dose response of Lymphoma 2 (lymphoid leukemia) to 8-azaguanine. Treatment in single daily doses, starting 2 days after inoculation. Initial inoculum was small, resulting in longer survival time than usual. The figures in parentheses indicate increase in survival time in per cent.

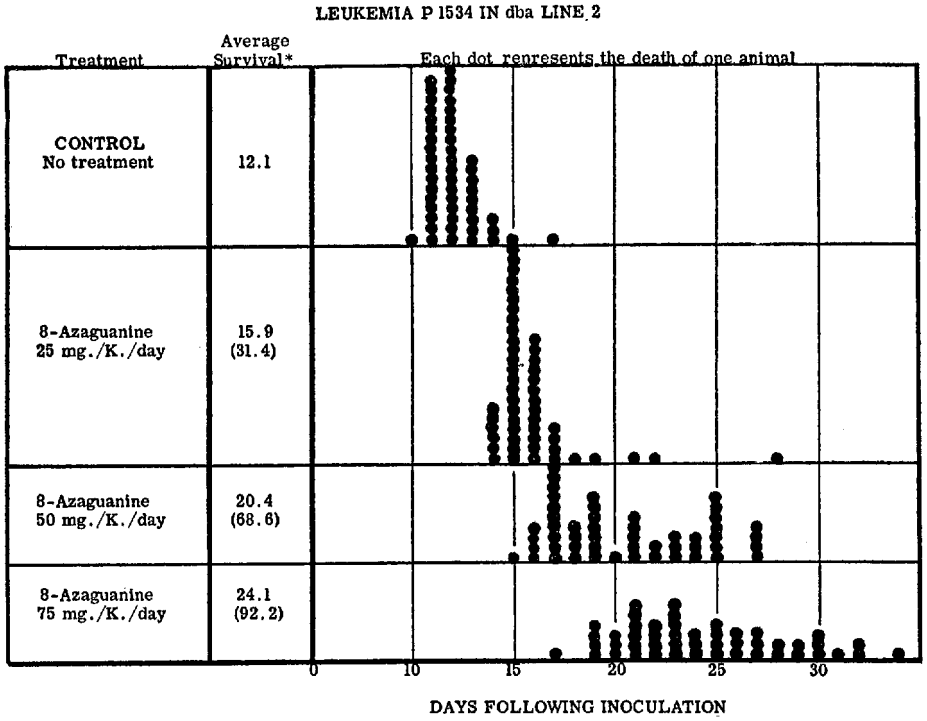

CHART 4.—Dose response of Leukemia P 1534 (lymphoid leukemia) to 8-azaguanine. Treatment in single daily doses starting 2 days after inoculation. The figures in parentheses indicate increase in survival time in per cent.

times within the various dose levels for both Lymphoma 2 and leukemia P1534 show the same relationships as in tumor 755. Some of these data are presented in Charts 3 and 4.

Reversal experiments.—Earlier attempts to cause the reversal by guanine administration of the inhibition of 8-azaguanine in tumor E 0771 were inconclusive (9), owing to the small amount of guanine employed. Law (10) was successful in showing release of 8-azaguanine inhibition in leukemia L 1210 when guanine HCl was administered subcutaneously in peanut oil or gum acacia. We have obtained a definite, though incomplete, release of inhibition in tumor 755 by the subcutaneous injection of guanine at 330 mg/kg/day, given just before administration of the inhibitor (Table 3). The same type of results was obtained by the inclusion of guanine in the food at 10 mg/gm of diet, but the responses were more irregular.

The reversal type of experiment is essential to an understanding of the mechanisms involved in any type of inhibition. The results that were obtained are in line with those found with Tetrahymena and suggest that the 8-azaguanine is interfering with the incorporation of guanine by the tumor cells. It is perhaps not surprising that the action of 8-azaguanine could not be completely

TABLE 3

ADENOCARCINOMA 755 IN C57 BLACK MICE*

Treatment	No. animals	Av. tumor wt. (gm.)	P (probability)†
Control	14	6.02	0.43
Guanine (330 mg/kg/day)	14	5.12	
8-Azaguanine (32.5 mg/kg/day)	17	1.85	0.013
8-Azaguanine (32.5 mg/kg/day) Guanine (330 mg/kg/day)	15	3.50	
8-Azaguanine (65 mg/kg/day)	18	0.49	0.032
8-Azaguanine (65 mg/kg/day) Guanine (330 mg/kg/day)	16	1.69	

* Experiment terminated 36 days after inoculation.

† Based on a chi-square analysis of the number of cases in each series falling above and below the mean value for the pair.

counteracted by guanine, in contrast to the results obtained with Tetrahymena, when one considers the solubilities of the metabolite, the large amounts that must be given, and the probable rate of excretion or destruction by the tissue enzymes.

Treatment at various tumor ages.—Sugiura *et al.* (18) obtained striking inhibition of the growth of tumor E 0771 with 8-azaguanine when therapy was started 24 hours after inoculation. When therapy was delayed until 6 days after inoculation they state that no inhibition occurred. Sokoloff *et al.* (16) have reported, in abstract form, that 8-azaguanine treatment of a rat carcinoma causes growth inhibition if treatment is started early but is inactive if treatment is delayed. These obser-

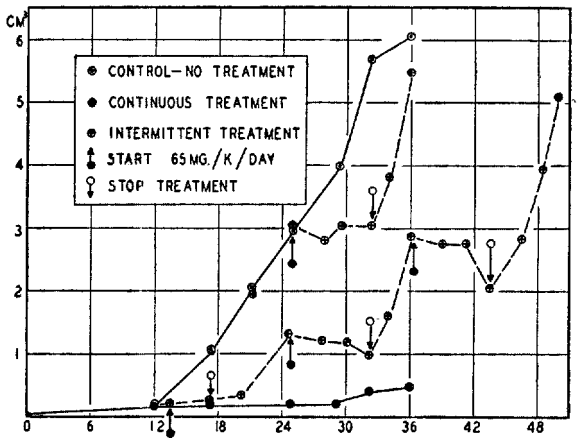

CHART 5.—Intermittent as compared with continuous treatment with 8-azaguanine. Treatment was in single daily doses. Twenty animals in each of the four series.

vations immediately raise the question as to whether the inhibitor is able to act on implanted tumor tissue only before it has become adjusted to its host. To answer this question, experiments were conducted with tumor 755 and Lymphoma 2. It had already been shown by our previous experiments with tumor E 0771 (9) that inhibition resulted when therapy was delayed until 6 days after inoculation, and Gellhorn *et al.* (4) had demonstrated inhibition of this tumor when therapy was delayed until 9 days after inoculation. In addition, they had shown that 8-azaguanine therapy was effective in producing inhibition of growth in tumor 755 when started as long as 22 days after inoculation and in tumor RC 7 days after inoculation.

Four groups of twenty each of C57 black mice were inoculated with tumor 755. Because of the nature of this experiment, caliper measurements were made of the tumors in order that growth might be followed. The control group was not treated. One group was treated with 8-azaguanine at 65 mg/kg/day in single daily doses beginning on the 13th day after inoculation. This treatment was continued until the 36th day, when this group, along with the controls, was sacrificed for wet weight measurements. The course of tumor development in these two groups is shown in Chart 5.

A third group was given 8-azaguanine from the 13th to the 17th day following inoculation. Treatment was then withheld until the 25th day, when it was again instituted and continued until the 32d day. On the 36th day the final course of treatments was begun and continued until the 43d day. The course of the tumor development in this group receiving intermittent treatment is shown in Chart 5. The tumors in the fourth group were allowed to develop until the 25th day after inoculation before treatment was given. They averaged, at this time, approximately 3 cc. Even after this period of growth and adjustment to the host, the treatment with 8-azaguanine was effective in producing inhibition.

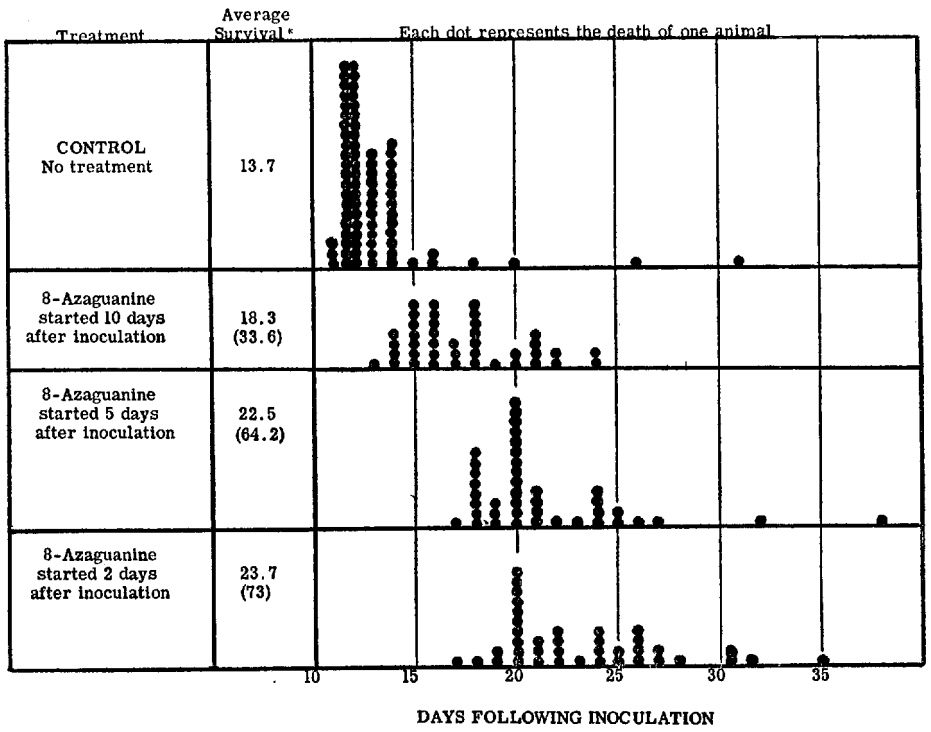

CHART 6.—Effect of treatment with 8-azaguanine (75 mg/kg/day in single doses) after establishment of leukemic condition for varying periods of time. Note that there is an inhibitory effect of the treatment, as judged by survival, even when treatments are withheld until less than 4 days before the average time of death. The figures in parentheses indicate increase in survival time in per cent.

Initiation of 8-azaguanine therapy after varying periods of time following inoculation of F_1 A/Street hybrids with Lymphoma 2 was carried out. The results are shown in Chart 6, where it can be seen that the compound has inhibitory effects even when given after the course of the disease is far advanced.

Molecular specificity.—If the tumor cells of the responsive types are similar to Tetrahymena in their ability to metabolize pre-formed guanine (and possibly in a requirement for guanine), then one would expect any changes in the substituent groups of 8-azaguanine to diminish or abolish the inhibitory action of such a compound. It was earlier found that substitution of an amino group for the hydroxyl in position 7 of 8-azaguanine (diaminazolo or 8-aza-2,6-diaminopurine) produced a compound with low inhibitory powers for Tetrahymena (6) and practically no inhibitory powers for tumor E 0771 (9).

It had been found that 8-azadenine (7-amino-1*H*-*v*-triazolo [*d*] pyrimidine) was much less inhibitory for Tetrahymena than was 8-azaguanine (8). The inhibition index of the former compound was 7, as compared to 0.02 for 8-azaguanine. This was to be expected in an organism which does not require (but is able to metabolize) pre-formed adenine. It was also expected that 8-azadenine would prove more toxic to mice than 8-azaguanine, owing to the reported ability of the mammal to metabolize adenine (2).

While it was found that 8-azadenine was much more toxic than 8-azaguanine and, unlike the latter compound, caused restriction in food consumption, it was possible to carry out experiments

on tumor inhibition provided doses no larger than 32 mg/kg/day were given. The effectiveness of 8-azadenine was of doubtful significance, since its P value, when compared to the control, was 0.25 (Table 4).

TABLE 4

ADENOCARCINOMA 755 IN C57 BLACK MICE*

Treatment	No. animals	Av. tumor wt. (gm.)
Control	28	2.02
8-Azaguanine (65 mg/kg/day)	20	0.035
8-Azaguanine (32.5 mg/kg/day)	20	0.177
8-Azadenine (32.5 mg/kg/day)	14	1.078
2-Mercapto-8-Azadenine (65 mg/kg/day)	10	2.115

* Experiment terminated 24 days after inoculation.

The addition of an SH group at position 2 of 8-azadenine (5-mercapto-7-amino-1*H*-*v*-triazolo [*d*] pyrimidine) completely destroys the inhibitory action for both Tetrahymena (8) and tumor 755 (Table 4), although this compound produced skin eruptions in the host.

DISCUSSION

In our previous report on the inhibitory action of 8-azaguanine (guanazolo) on certain mouse tumors, the suggestion was offered that a fundamental qualitative biochemical difference might exist between these neoplastic cells and normal cells. Based on the fact that the first three tumors tested were inhibited by 8-azaguanine, it was further suggested that this effect might be found to have widespread, if not universal, distribution among the various types of malignancies. If this were found to be the case, it would fulfill the postulate of Greenstein (5) that some fundamental qualitative biochemical deviation from normal exists in all tumors. It soon became apparent that the inhibitory effect of 8-azaguanine was by no means universal. To our knowledge, no tumor of the sarcoma series has been found to be inhibited by 8-azaguanine. However, it is also quite apparent that a large series of tumors is inhibited by this compound. In addition to the mouse tumors inhibited (Table 1), responses have been found with a rat carcinoma (16), the Brown-Pearce carcinoma of the rabbit (4), and the virus-induced plant tumor of the buttercup and tobacco (12). The plant tumor inhibition studies are of considerable interest, inasmuch as these tumors are grown in a synthetic culture medium and the inhibitions were found to be completely reversed by guanine. Moreover, when the inhibition index is calculated from the data given, it turns out to be similar to that found in the Tetrahymena studies (9).

Most of the investigations of the metabolite–anti-metabolite type in relation to neoplasms have used the quantitative metabolic differences which exist between these abnormal cells and the normal tissues as a point of departure. It is reasoned that, in view of the more rapid rate of growth (and therefore metabolism) of the tumor cells, as compared to most of the host cells, tumor cells should be inhibited by concentrations of analogs of normally required metabolites which are below the level of serious toxicity to the host tissues. This approach has produced certain promising results, as in the case of anti-folics. Had this line of reasoning been applied to purine anti-metabolites, one would have anticipated, from what is known of mammalian purine utilization, that an anti-adenine might show tumor inhibition while an anti-guanine would be inert. This is not the case, however, in a number of tumors where the 8-azadenine is found to be only slightly inhibitory, while 8-azaguanine produces marked carcinostasis. The fact that the 8-azaguanine inhibition is reversed by guanine is strong evidence that this compound acts as it does in the microörganisms—i.e., by competing in some way with guanine. The hypothesis that these tumor cells differ from normal tissues in that they are able to utilize pre-formed guanine is the most plausible yet offered to explain these observations. That the compound produces secondary effects on the tumors through its action on the host tissues is inconsistent with the observations that not all tumors are affected. This was nicely shown by Sugiura *et al.* (18), when they found that 8-azaguanine had no inhibitory effect on Sarcoma 180, even when transplanted into the normal host (C57 black) for E 0771.

The hypothesis earlier advanced for the extreme activity of 8-azaguanine as an inhibitor of Tetrahymena (6), namely, that the inhibitor is metabolized by the guanine enzyme systems in place of guanine and is therefore incorporated into the nucleoproteins, has received support from the observations of Bennett *et al.* (1) and Mitchell *et al.* (11). They found that 2-C^{14}-labeled 8-azaguanine, when administered to normal CFW mice and to C57 black mice bearing tumor E 0771, was largely excreted in the urine. Some activity was found, however, in the ribonucleic acid isolated from various tissues and from the tumors. Using paper chromatographic methods, they claimed evidence for the incorporation of 8-azaguanine, as such, into the RNA of both tumor tissues and non-tumor tissue, particularly in the jejunum. These results seem to indicate that there is no preferential selection of 8-azaguanine by these tumor cells.

However, it must be pointed out that the results of Shapiro *et al.* (15) can certainly be best interpreted as selective action of 8-azaguanine on the Brown-Pearce carcinoma in the rabbit. They showed that the mitotic index for tumor cells was markedly reduced by the administration of 8-azaguanine, while the mitotic indices of the intestinal epithelium and of the testis of the same animal were not affected.

It seems clear that the final establishment of a qualitative biochemical difference between any neoplastic cells and normal cells is of enormous importance to our understanding of the fundamental principles of malignancies. The bulk of the evidence is, in our opinion, consistent with the theory that such a difference has been shown by the use of 8-azaguanine. Additional carefully executed experiments with labeled compounds will be needed for definitive answers. Such studies are now under way in this laboratory.

SUMMARY

1. The guanine analog, 8-azaguanine (guanazolo) has been found carcinostatic for adenocarcinoma 755 and Lymphoma 2, thus confirming earlier observations (4, 9, 10).

2. Leukemia P1534 in dba mice and spontaneous adenocarcinoma of Swiss mice were also inhibited by the administration of 8-azaguanine.

3. Sarcoma 37 in C57 black mice and melanoma S 91 in dba mice were not inhibited by the administration of 8-azaguanine.

4. The ineffectiveness of 8-azaguanine in the inhibition of Sarcoma 180 and Lymphosarcoma 6C3HED was confirmed (4, 17, 18).

5. Dose-response tests with 8-azaguanine on tumor 755 and Lymphoma 2 show that these tumors respond in a regular manner to increasing concentrations of the compound.

6. Definite, though incomplete, reversal of the inhibition of the growth of tumor 755 produced by 8-azaguanine was obtained with guanine.

7. It was shown that tumor 755 and Lymphoma 2 are inhibited by 8-azaguanine even after the implants have been established for relatively long periods of time.

8. The adenine analog, 8-azadenine, was found to be relatively inactive as an inhibitor for tumor 755, while 2-mercapto-8-azadenine was inert.

9. The available evidence is discussed for its bearing on the question of the existence of a fundamental biochemical difference between certain tumor cells and normal mammalian cells.

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A Study on the Biologic Activity of a Transplanted Granulosa-Cell Tumor in Castrate C57 Mice*

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The purpose of this report is to present the results of a study on the growth of a specific transplanted granulosa-cell tumor and the progressive effects it has on the host tissues.

METHODS

Fifty-three mice, 3–4 months old, of the C57 black strain were used, 18 females and 35 males. All the animals were castrated at the beginning of the experiment and were maintained on Laboratory Chow and water *ad libitum*. They were weighed weekly. Eleven females and 21 males each received a transplant of a small fragment of a granulosa-cell tumor (18C57), which was introduced subcutaneously with a No. 15 gauge trocar 1–6 days following castration. The tumor used in this study was in its fifth transfer generation and showed no change from its original histological structure (Fig. 1). Seven females and fourteen males which did not receive tumor transplants were used as controls. All control animals were sacrificed 60 days after castration. Following transplantation each animal was examined daily until a palpable tumor nodule was present. Daily vaginal smears were taken from 1 week after castration until the time the animals were sacrificed. All tumor-bearing animals were sacrificed at intervals of from 62 to 100 days following transplantation. At autopsy the tumors and visceral organs were weighed and then preserved in Bouin's fixative for histological study. Gross mounts of the mammary glands were made.

OBSERVATIONS

A tumor was considered a "take" when a distinct, firm nodule approximately 5 mm. in diameter could be palpated. The first nodule did not occur until the 18th day following transplantation. By the 28th day after transplantation 22 "takes" had occurred. At the end of the 52d day "takes" were palpated in all the animals with transplants. There was no significant difference between the onset of the tumor "takes" of the males and females, although the tumor grew more rapidly in the males than in the females.

At autopsy, the animals with tumors had dilated and slightly heavier hearts than did the controls (Tables 1 and 2).

All the livers of the tumor animals, except those from the two with the smallest tumors, were en-

TABLE 1

AVERAGE ORGAN AND BODY WEIGHTS OF CASTRATED MALE MICE (C57) BEARING A TRANSPLANTED GRANULOSA-CELL TUMOR

	Controls	Group 1	Group 2	Group 3	Group 4
Number of mice	14	3	6	6	6
Tumor weight (range) (gm.)		1.13 2.90	3.13 3.95	4.04 4.81	5.00 6.26
Body weight (gm.)	24.00	28.30	28.60	27.30	30.70
Liver (gm.)	1.13	1.68	1.82	2.17	2.28
Heart (mg.)	110.0	120.0	120.0	180.0	150.0
Spleen (mg.)	140.0	270.0	290.0	330.0	400.0
Kidneys (mg.)	270.0	320.0	280.0	280.0	270.0
Submaxillary glands (mg.)	90.0	90.0	70.0	80.0	90.0
Adrenal glands (mg.)	5.6	6.3	5.8	5.3	5.4
Seminal vesicles (mg.)	26.0	27.7	38.4	24.4	25.4

TABLE 2

AVERAGE ORGAN AND BODY WEIGHTS OF OVARIECTOMIZED FEMALE MICE (C57) BEARING A TRANSPLANTED GRANULOSA-CELL TUMOR

	Controls	Group 1	Group 2	Group 3
Number of mice	7	4	3	4
Tumor weight (range) (gm.)		1.56 2.56	4.30 4.90	5.28 6.58
Body weight (gm.)	22.50	23.30	27.70	30.10
Liver (gm.)	1.20	1.41	2.43	3.01
Heart (mg.)	110.0	120.0	160.0	250.0
Spleen (mg.)	140.0	240.0	390.0	460.0
Kidneys (mg.)	270.0	270.0	290.0	380.0
Submaxillary glands (mg.)	110.0	80.0	70.0	110.0
Adrenals glands (mg.)	5.6	4.9	6.9	5.9
Uterus (mg.)	14.6	27.6	52.6	78.1

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larged and congested, and their surfaces presented a peculiar mottled appearance. This congestion and mottling was more marked in animals with large tumors, and mice with tumors over 5 gm. in weight had livers weighing 2–3 times more than those of the controls (Tables 1 and 2). Microscopically, the livers of the tumor-bearing animals showed dilatation of the blood sinusoids, varying from a slight to a very marked degree (Figs. 3–6). This progressive dilatation of the sinusoids was correlated with the increase in tumor weight. The sinusoidal dilatation set in rather rapidly and was noted in all livers except in those of one female and one male which had the smallest tumors—1.56 gm. and 1.13 gm., respectively. In all animals with tumors over 3.00 gm., moderate to extreme dilatation was noted, with most of the liver substance displaced by large, dilated blood sinuses. Although there was an increased blood volume in the tumor-bearing animals, the progressive increase in body weight was approximately equal to the increase in tumor weight (Tables 1 and 2). The spleens of these animals increased progressively in size and weight with the tumor weight (Tables 1 and 2) and microscopically showed vascular congestion. No increase in hemopoiesis was noted.

There was no significant change in the weight of the kidneys in the tumor animals as compared to those in the control animals (Tables 1 and 2). Microscopic examination of the kidneys of the castrated male control animals revealed that they were of the male type in which the greater percentage of Bowman's capsules have a parietal epithelium consisting of cuboidal cells (Fig. 7). The parietal epithelium of the remaining small percentage of capsules consisted of squamous cells. In four female control animals the number of the female type was slightly predominant, while in three female controls there was approximately an equal number of both types. The kidneys of the male and female tumor-bearing animals had capsules of the female type, with a parietal epithelium consisting of squamous cells, while only a small number of male-type capsules was seen (Fig. 8).

Histological examination of the submaxillary glands revealed that all the controls had an altered female type, which is not uncommon in castrated male and female as well as intact female mice of the C57 strain (unpublished data). This altered type is similar to that produced by injecting progesterone into castrated male and female mice (3). The lining epithelium of the terminal tubules of these altered female type glands consisted of columnar cells containing little or no secretion granules. The nucleus was situated at the basal portion of each cell (Fig. 9). Eight male animals and three female animals with tumors of less than 3.95 gm. in weight had submaxillary glands of this altered female type. All the animals with tumors weighing 3.95 gm. or more had glands which exhibited the characteristic features of the estrogen-stimulated female type described by Lacassagne and Fekete (2, 5). The lining epithelium of the terminal tubules of these glands consisted of cuboidal cells with the nucleus located in the center of the cell (Fig. 10).

No change was noted in the adrenal or pituitary glands. The X-zone of the adrenal glands was either extremely degenerated or absent. Dilatation of the cortico-medullary sinuses of the adrenal gland was not noted in any animal in this study.

The seminal vesicles and prostate glands of the male tumor-bearing animals were atrophic.

The weight of the uteri of the female animals with tumors increased progressively as the weight of the tumor increased (Tables 1 and 2). The females with the largest tumors had uteri 4–5 times heavier than those of the controls. Histologically, the uteri of the control females were of the castrate type, while the uteri of all the tumor-bearing females showed varying degrees of estrogenic stimulation (Fig. 15). The uteri of the animals with the largest tumors showed the most marked stimulation. No progesterone effect was noted. Likewise, the vaginal mucosa of these animals was heavily cornified, whereas the vaginal mucosa of the animals with smaller tumors showed only a slight increase in cell layers when compared to the atrophic epithelium of the castrated controls (Figs. 11–14).

The first estrous-type vaginal smear was obtained from a female animal 51 days after its tumor was first palpated. The animal was sacrificed at that time. Its uterus weighed 60.0 mg. and the tumor 4.30 gm. This same animal had the first cornified vaginal epithelium. In the succeeding tumor-bearing female animals it was observed that they would have an estrous-type smear for 3 or more days and then have a diestrous-type smear for a period of 5 days. Several animals were permitted to run such "artificial" cycles before being sacrificed. The uteri of these females weighed from 66.0 to 82.0 mg. Careful examination of these animals at autopsy revealed no gross evidence of ovarian tissue. It is also unlikely that there was any ovarian tissue present, as these animals previously had diestrous vaginal smears for a period of 90 days or more.

Gross mounts of the mammary glands revealed varying degrees of stimulation correlated with the tumor weight (Figs. 16–22). The mammary glands of the males with tumors weighing from 2.90 to

5.00 gm. showed evidence of early stimulation; their ducts were dilated, and end buds were present. Three males of the above group with tumors weighing 2.45, 3.13, and 3.42 gm. were exceptions in that their mammary glands showed no signs of stimulation. In males with tumors weighing from 5.00 to 6.26 gm., the mammary glands showed beginning alveolar development. The glands of the females with tumors weighing from 2.25 to 4.43 gm. showed slight stimulation, and in females with tumors from 4.43 to 6.58 gm. there was alveolar development. Of the latter group, the structure of the female glands was much more extensive than that of the males showing alveolar development.

DISCUSSION

A close correlation existed between the size and weight of the granulosa-cell tumor and the amount of estrogenic substance secreted. Also, a close correlation occurred between the tumor size and weight and the severity of hypervolemic changes. Therefore, it appears that the response in both instances depends on the size and weight of the tumor. This is contrary to the findings of Geist and his co-workers (4), who studied parenchymatous lutein tumors of mice produced by roentgen irradiation. They concluded that the biologic activity of these tumors, as expressed by the effect produced upon the uterus and vagina, did not depend upon the size of the tumor or the extent of luteinization.

The kidney, submaxillary and mammary glands, in addition to the uterus and vagina, served as useful indicators of estrogenic secretion by the tumor. The kidneys of the male control animals did not revert to the characteristic female type upon castration, and the kidneys of the female control mice did not retain the characteristic female type following ovariectomy, as might be expected. Crabtree (1) found that after castrating male mice there was a decrease in the percentage of Bowman's capsules lined with cuboidal cells to approximately the female level for animals of the same age, and also noted that accompanying this decrease there was a regeneration of the X-zone or androgenic zone of the adrenal. Due to the fact that the X-zone of the adrenal in all the mice of this experiment was extremely degenerated or absent, one might suspect this to be an important factor in the explanation as to why the castrated and ovariectomized controls had kidneys which tended to be or were the male type. However, this occurrence served a useful means by indicating that a tumor weighing only 1.13 gm. secreted a sufficient amount of estrogenic substance to cause the kidney to assume the characteristic female type. The submaxillary gland findings were analogous to those of the kidneys. The epithelial cells of the terminal tubules of the glands in all the controls were columnar instead of the usual cuboidal found in castrates. This occurrence also served a useful means by indicating that a tumor weighing 2.56 gm. in the female and 3.95 gm. in the male secreted a sufficient amount of estrogenic substance to cause the submaxillary glands to become the characteristic estrogen-stimulated female type.

Because the females with tumors weighing 4.43 gm. or more showed cyclic vaginal cornification, it appears that the estrogenic secretion of the large tumors was rhythmic, possibly being governed by the pituitary.

Trentin (7) made single injections of estradiol benzoate in oil into ovariectomized mice of eleven inbred strains to determine the amount necessary to cause 50 per cent of a strain to produce cornified vaginal smears. He found the mice of the C57 strain were the most sensitive; a single injection of 0.06 μg. produced the desired effect. Using this finding, one may estimate that a tumor weighing 4.30 gm. was secreting an amount of estrogenic substance with a stimulating effect approximately equal to that which 0.06 μg. of estradiol benzoate has on the vaginal mucosa.

There was no evidence that the tumor produced androgens. The hypervolemic changes associated with granulosa-cell tumors are not caused by the estrogenic substance secreted by the tumor, for experiments on sustained administration of large doses of stilbestrol and natural estrogens failed to elicit hypervolemia in mice (6). Similar changes are also seen in mice with nonestrogen-producing tumors.

SUMMARY

1. A study of a transplanted granulosa-cell tumor in C57 castrate mice revealed a close correlation between the size and weight of the tumor and the quantity of estrogenic substance secreted.

2. It was estimated that a tumor weighing 4.30 gm. was secreting an amount of estrogenic substance with a stimulating effect approximately equal to that which 0.06 μg. of estradiol benzoate has on the vaginal mucosa of C57 mice.

3. A close correlation was also found to exist between the size and weight of the tumor and the severity of hypervolemic changes which occurred in the tumor-bearing mice.

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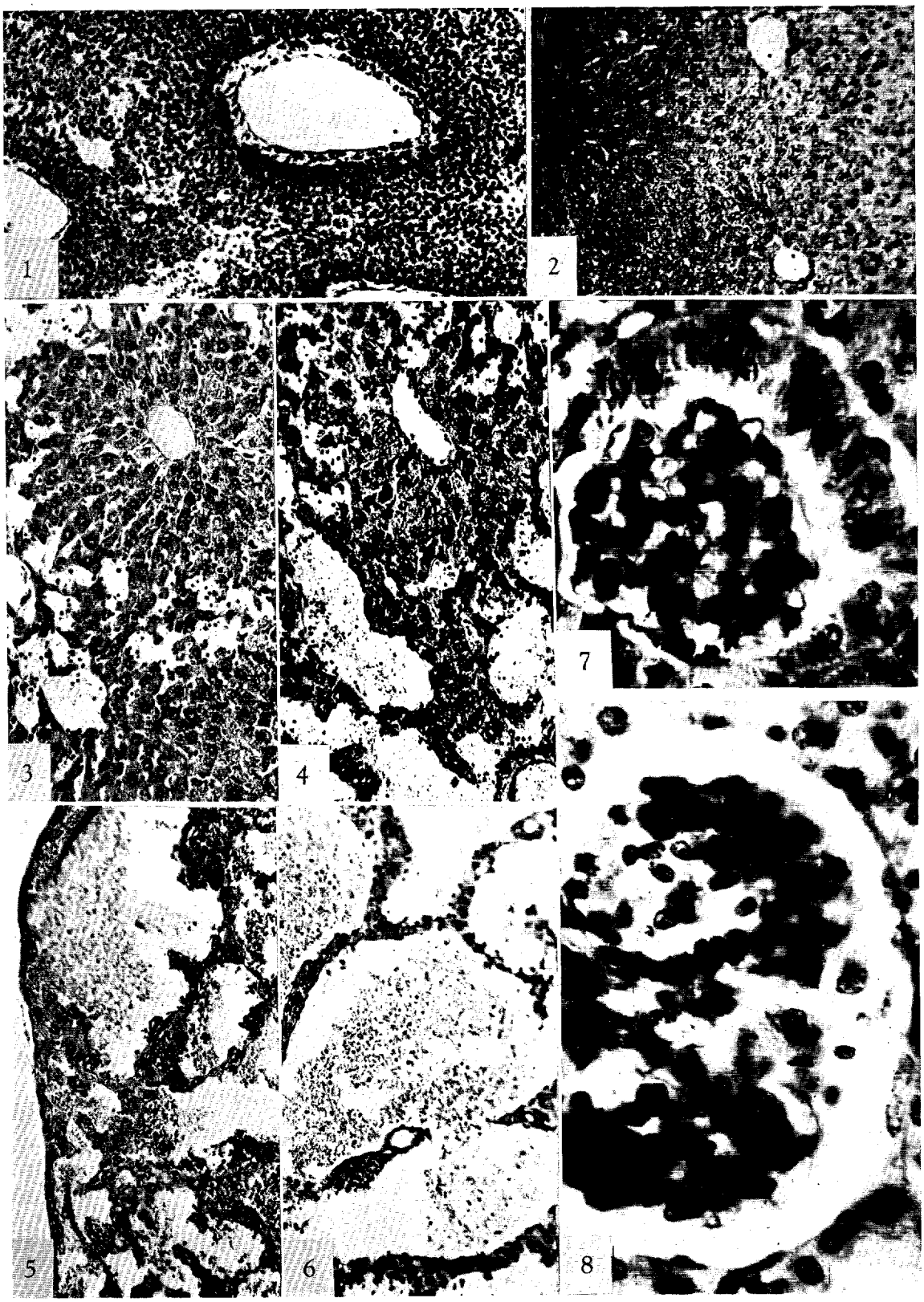

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FIG. 1.—Representative histological section of the granulosa-cell tumor showing the typical architecture. Mag. ×100.

FIG. 2.—Liver of an ovariectomized female control mouse. Mag. ×100.

FIG. 3.—Liver showing early (2+) sinusoidal dilatation from a castrated male mouse with a 3.95-gm. tumor. Mag. ×100.

FIG. 4.—Liver showing advanced (3+ to 4+) sinusoidal dilatation from an ovariectomized female mouse with a 5.39-gm. tumor. Mag. ×100.

FIG. 5.—Liver showing marked (4+) sinusoidal dilatation with rupture of the liver capsule imminent. From an ovariectomized female mouse with a 5.28-gm. tumor. Mag. ×100.

FIG. 6.—Liver showing extreme sinusoidal dilatation with preservation of the small bile ducts. Section from an ovariectomized female mouse with a 6.58-gm. tumor. Mag. ×100.

FIG. 7.—Renal corpuscle from the kidney of a castrated male control mouse. Cuboidal epithelial cells are present in the parietal layer. Mag. ×600.

FIG. 8.—Renal corpuscle from a kidney of an ovariectomized female mouse with a 6.58-gm. tumor. Squamous epithelial cells are present in the parietal layer. Mag. ×600.

FIG. 9.—Submaxillary gland from an ovariectomized female control mouse. The terminal tubular epithelium is the altered female type. Mag. ×1,000.

FIG. 10.—Submaxillary gland from a castrated male mouse with a 6.26-gm. tumor. The terminal tubular epithelium is the characteristic female type. Mag. ×1,000.

FIG. 11.—Atrophied vaginal mucosa from an ovariectomized female control mouse. Mag. ×450.

FIG. 12.—Vaginal mucosa from an ovariectomized female with a 1.56-gm. tumor. The mucosa shows early signs of stimulation with an increase in cell layers. Mag. ×450.

FIG. 13.—Vaginal mucosa from an ovariectomized female with a 4.43-gm. tumor. The thickened mucosa indicates moderate stimulation. Mag. ×450.

FIG. 14.—Cornified vaginal epithelium from an ovariectomized female mouse with a 5.62-gm. tumor. Mag. ×450.


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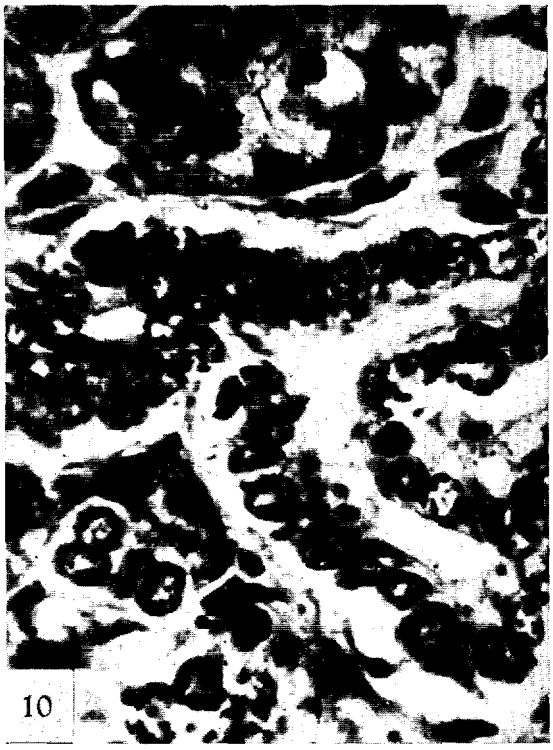
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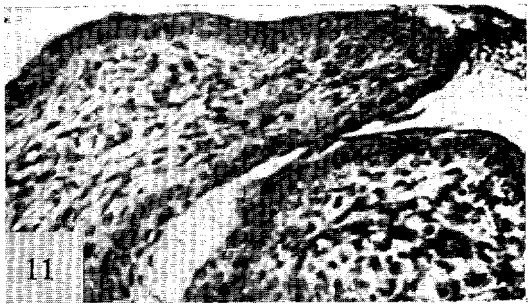
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FIG. 15.—Castrate uterus from a female control mouse (insert) and a section of a uterus showing marked stimulation from an ovariectomized female mouse with a 4.43-gm. tumor. Same magnification. Mag. ×55.

FIG. 16.—Atrophied mammary gland from a castrated male control mouse. Mag. ×55.

FIG. 17.—Mammary gland from a castrated male mouse with a 2.90-gm. tumor. Early signs of stimulation are indicated by dilated ducts and end buds. Mag. ×55.

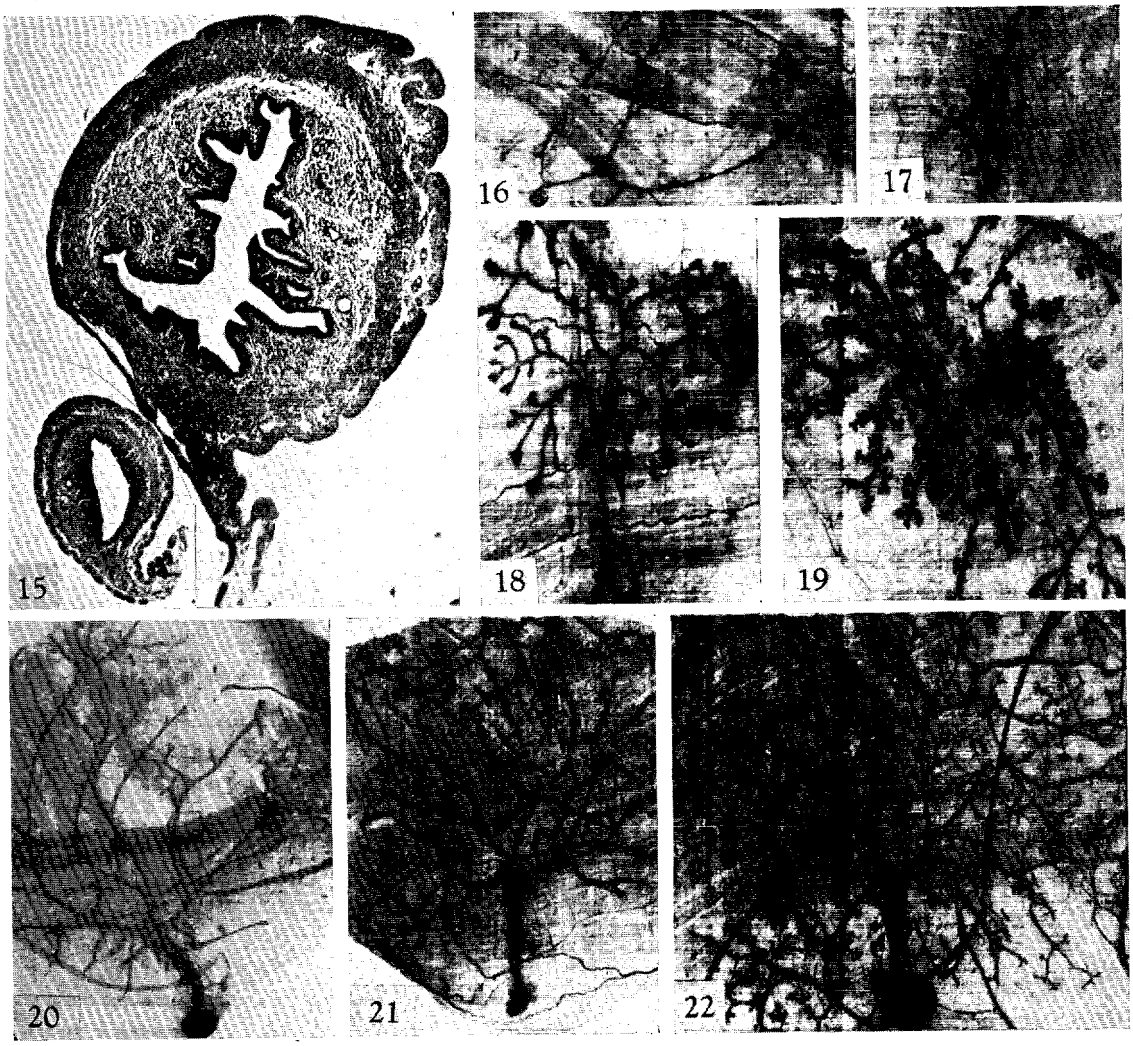
FIG. 18.—Mammary gland from a castrated male mouse with a 3.34-gm. tumor. The increased growth of the duct system indicates moderate stimulation. Mag. ×55.

FIG. 19.—Mammary gland showing beginning alveolar development from a castrated male mouse with a 5.00-gm. tumor. Mag. ×55.

FIG. 20.—Atrophied mammary gland from an ovariectomized female control mouse. Mag. ×55.

FIG. 21.—Mammary gland from an ovariectomized female with a 2.56 gm. tumor. Early signs of stimulation are indicated by dilated ducts and end buds. Mag. ×55.

FIG. 22.—Mammary gland showing beginning alveolar development from an ovariectomized female mouse with a 5.62-gm. tumor. Mag. ×55.



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A Study of the Metabolism of 20-Methylcholanthrene*

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INTRODUCTION

Although 20-methylcholanthrene has been widely investigated with respest to its role as a carcinogen, the information available as to its distribution and its metabolism is relatively sparse. In previous studies with regard to these aspects, the usual spectroscopic methods have been employed. Chalmers and Peacock (3), using the fluorescence method, showed that the compound could be detected in the bile of mice, 1–2 hours following intravenous injection of an aqueous colloid; no original hydrocarbon could be detected after 3 hours. Chalmers (2) also found that the fluorescent spectrum of urine and feces 24 hours after intravenous injection showed no specific bands of methylcholanthrene. Simpson and Cramer (10) have found that 10 days after painting the skin with a benzene solution of methylcholanthrene no specific spectrum of the hydrocarbon could be found in any layers of the skin. Also, Lorenz and Shimkin (9) found that 4 days after intravenous injection the carcinogen could be detected in the lungs, as indicated by its fluorescent properties, but that at the end of 1 week all the material had disappeared.

More recently, Heidelberger, Jones, and coworkers (7, 8) have studied the distribution and metabolism of dibenzanthracene employing a C^{14}-labeled hydrocarbon. It was found that 8–9 months after subcutaneous injection, i.e., when the tumors appeared, 1–5 per cent of the original hydrocarbon was present in the tumor, and a general distribution of C^{14} throughout all tissues was noticed. In view of these more elaborate experiments with dibenzanthracene, it was decided that a similar investigation of the metabolism and distribution of methylcholanthrene labeled with C^{14} would be of interest. The preparation of this carcinogen labeled in the 11-position is reported elsewhere (4).

EXPERIMENTAL METHODS

Strain "A" male mice were used throughout the experiment. They received subcutaneous injections of 1.0 mg. of methylcholanthrene in 0.05 ml. of tricaprylin, in the right axilla. The specific activity of the methylcholanthrene was 135,000 cts/min/mg. For elimination studies, each mouse was injected and placed in a glass metabolism cage. Urine and feces were collected daily for a 7-day period, and the cages were washed with benzene and water. An aliquot of urine specimens was plated and counted directly (1). The feces, tissues, or organs were dried *in vacuo* and oxidized by a modification (6) of the method of Skipper (11); the entire sample was oxidized in all cases. The carbon dioxide was absorbed in aqueous sodium hydroxide and precipitated as barium carbonate. The solid was filtered, dried, weighed, plated according to the method of Dauben, Reid, and Yankwich (5), and counted either with a thin-window Geiger Müller tube or in a proportional flow counter (Nucleometer). The over-all error in each determination is less than 5 per cent.

DISTRIBUTION OF RADIOACTIVITY

No detectable activity was found in respired carbon dioxide, and this was also true in the case of dibenzanthracene (7). Liver, gastrocnemius muscle, and mesenteric fat were chosen as representative tissues for study of the distribution of radioactivity (Table 1). The tissues were removed at periodic intervals and assayed as previously described. In all cases, less than 1 per cent of the injected dose was present in the tissue, and there was no significant difference in the distribution of activity among these three tissues. In some cases, Heidelberger and Jones found a higher percentage of radioactivity in the liver when their carcinogen was administered as a colloid. Occasionally, lymph nodes and whole blood were removed and found to contain approximately the same activity as liver, muscle, and fat.

RETENTION AND ELIMINATION OF RADIOACTIVITY

Urine and feces.—The rate of elimination of radioactivity in the feces was fairly rapid for the first 2 days and then leveled off for the remainder of the collection period (Chart 1). The rates are self-consistent for each individual mouse, although the variation among mice was considerable. At the

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end of 1 week after injection, 9–25 per cent of the injected activity had been eliminated in this manner.

Urinary excretion (Chart 2) is small compared to the amount found in the feces—only about one-tenth as much being excreted by this route. No attempt was made to determine whether the urinary or fecal activity represented unchanged methylcholanthrene.

Site.—A series of mice was given a single subcutaneous injection of methylcholanthrene, sacrificed periodically, and the entire region of the site of injection was oxidized and assayed. The results of these experiments are shown in Chart 3. These data indicate a fairly rapid elimination during the first week, after which a constant slower rate is maintained. At the end of 8 weeks, approximately 20 per cent of the activity remains at the site of injection. The last point on the curve represents the average percentage of activity found in twenty tumors.

Tumor.—Three months after injection, there is an 85 per cent incidence of tumors (Table 2), of which 82 per cent were spindle-cell sarcomas. There was no significant difference between the rate of tumor formation in animals receiving radioactive methylcholanthrene and that in those receiving the nonradioactive carcinogen (Table 2). The amount of injected radioactivity found in these tumors averaged 7 per cent.

Metabolic degradation.—To compare the extent of degradation of methylcholanthrene with dibenzanthracene, tumors produced with the labeled carcinogen (see preceding paragraph) were chemically fractionated. The methods employed were the same as those described by Heidelberger, Kirk, and Perkins (8). The summary of this fractionation is shown in Chart 4. The results are expressed as percentage of the radioactivity in the original injection. Radioactivity was determined in each step, either by the direct plate technic or by oxidation.

The methylcholanthrene present in the tumor was determined by isotopic dilution technic. Inactive methylcholanthrene (20 mg.) was added to the homogenized tumor and isolated from the benzene solution. It was purified by repeated recrystallization from ligroin and ether. The hydrocarbon was then oxidized and the activity determined; the activity represented the methylcholan-

TABLE 1

PER CENT OF INJECTED DOSE IN TISSUE

Tissue	Experiment number	Days 1	2	3	4	5	6	7	Weeks 2	3	4	5	6	7	8
Liver	1	0.2	0.3	0.0	0.0			0.9	0.0	0.4	0.9	0.7	0.5	0.4	0.2
	2	1.0	0.4	0.5	0.4	0.2	0.2	0.2	0.1	0.0	0.0				
Fat	1	0.0	0.0	0.0	0.0			0.0	0.3	0.0	0.4	0.2	0.3	0.2	0.0
	2	0.0	0.0	0.0	0.2	0.0	0.0	0.1	0.0	0.0	0.1				
Muscle	1	0.0	0.1	0.0	0.0			0.7	0.3	0.1	0.1	0.0	0.0	0.0	0.0
	2	0.1	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0				

Experiment 1: 12 animals
Experiment 2: 10 animals

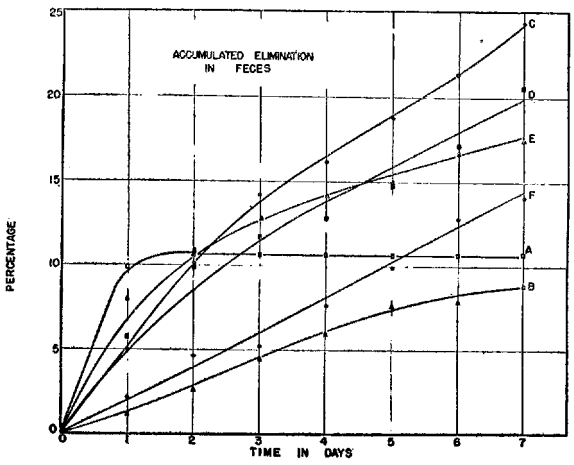


CHART 1.—Accumulated elimination in feces

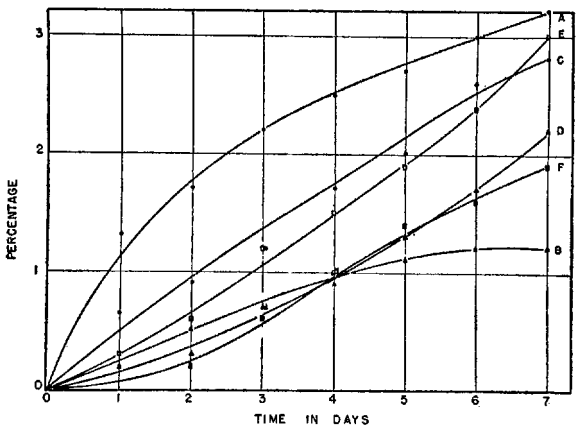


CHART 2.—Accumulated elimination in urine

threne which was not metabolized. Of the activity in the tumor, 10, 60, and 75 per cent was unmetabolized carcinogen. The amount of original hydrocarbon found in the tumor, 1–5 per cent, was of the same order of magnitude as that reported in the dibenzanthracene study.

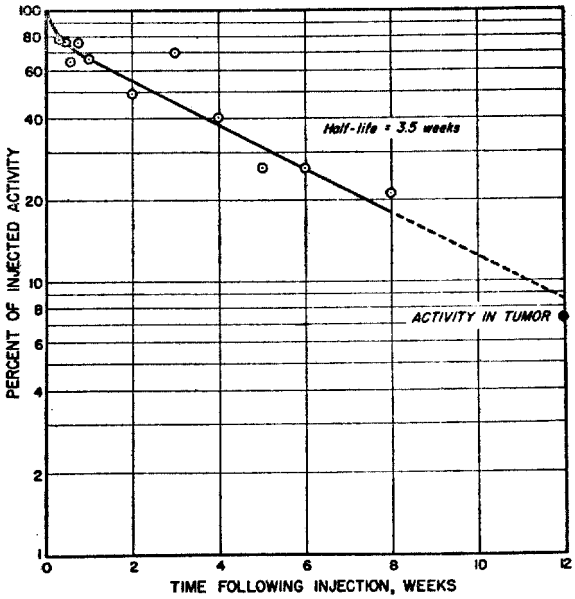


CHART 3.—Rate of disappearance of radioactivity from site of subcutaneous injection in tricaprylin.

ity of the activity which was introduced in the form of the hydrocarbon and which remains in the mouse is found at the site of injection. There appears to be no localization or concentration of C^{14} in any tissue or organ as indicated by liver, gastrocnemius muscle, and mesenteric fat.

The major route of elimination appears to be through the feces, while a lesser amount is lost in urinary excretion. There is an absence of radioactivity in the expired carbon dioxide. From examination of the instantaneous rate curves of the elimination by feces and urine (Chart 5), it can be seen that the former pathway appears to be composed of two distinct processes: a rapid process by which about 5 per cent of the injected C^{14} is eliminated in 3 days and a slower, more continuous pathway progressing at a rate of about 1.6 per cent per day. A similar and perhaps related metabolic process is found for the urinary excretion, by which C^{14} is eliminated at a rate of about 0.4 per cent per day. The results of Heidelberger and Jones on dibenzanthracene show that similar processes are involved with that hydrocarbon.

From the rate of disappearance of C^{14} from the site, it can be seen that about half of the activity has disappeared in about 4 weeks. Examination of the previously reported data on dibenzanthracene (7) indicates a value of about 10 weeks for the similar process. Such values are not unreasonable,

TABLE 2

INCIDENCE OF METHYLCHOLANTHRENE-INDUCED TUMORS

Dose, milligrams	Inactive methylcholanthrene		Active methylcholanthrene	
	Per cent incidence	Type of tumor	Per cent incidence	Type of tumor
1.0	80	Spindle-cell sarcoma	85	Spindle-cell sarcoma 1 Squamous-cell sarcoma 1 Adenocarcinoma 1 Adenocanthoma
0.5	75	Spindle-cell sarcoma 1 Lymphosarcoma	40	Spindle-cell sarcoma
0.1	5	Spindle-cell sarcoma	20	Spindle-cell sarcoma

Strain A male mice injected subcutaneously and sacrificed after 90 days. 20 animals in each group. Histology by Dr. Rosahn.

DISCUSSION

In the following discussion pertaining to the fate of methylcholanthrene when injected subcutaneously, most of the results are expressed as C^{14} activities and, as such, are not a direct measure of the carcinogenic hydrocarbon itself. Such data give no indication of the chemical nature of the marked carbon atoms but simply that a portion (or the whole) of the original compound is present in the fraction assayed.

It has been found that, when methylcholanthrene is employed as described above, the major-

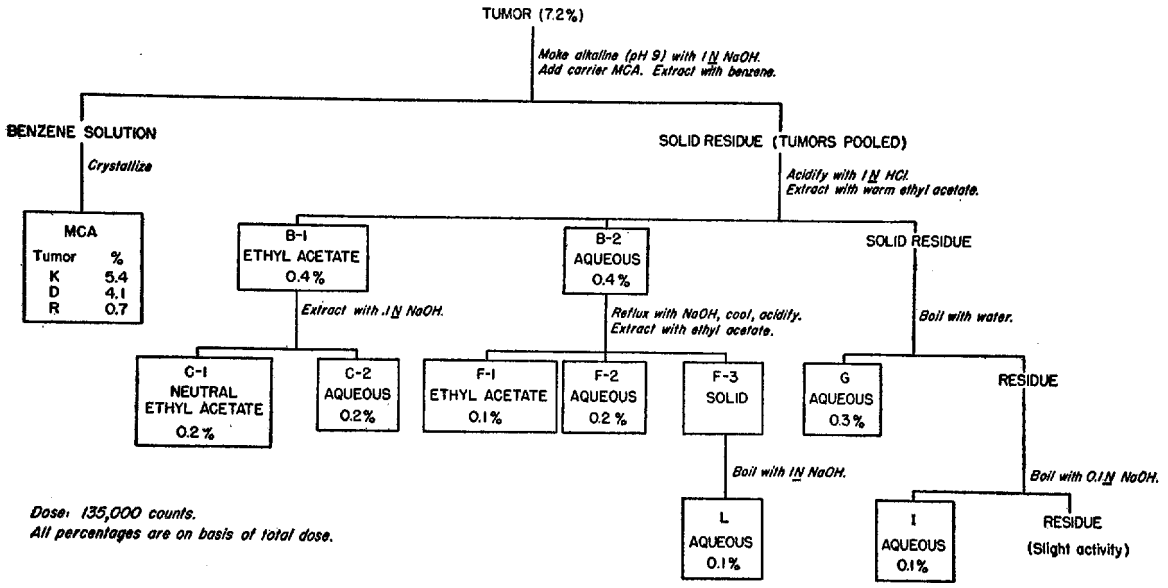
since it is known that the tumors produced by each carcinogen contain similar amounts of the original hydrocarbon and since the ratio of the latent periods for these compounds is about 2.5/1 (DBA/MAC). If the rate of disappearance from the site of the metabolic products is very rapid, as the work on dibenzanthracene would suggest, such half times indicate the actual metabolic rates of the carcinogens. However, if the rate of disappearance of metabolites is of the same order as the metabolic rate, then these values are composite rates of the two processes. Work is in prog-

ress to investigate this question more thoroughly. In view of the similar proportionality of the rate of disappearance from site and the latent periods of the carcinogens, it is suggestive that metabolism may well play a role in tumor production.

Extensive fractionation of the tumor showed a general but small distribution of radioactivity in all fractions, a result identical to that found with dibenzanthracene.

SUMMARY

20-Methylcholanthrene, labeled in the 11-position, has been administered subcutaneously in tricaprylin, and the distribution of radioactivity in the mice at various intervals has been determined. The radioactivity is eliminated mainly in the feces and to a lesser extent in the urine. An appreciable quantity of radioactivity is found in the tumors, part of which is the original hydrocarbon. There appears to be no localization of activity in any tissue or organ, the major amount remaining at the site of injection. The rate of elimination of C^{14} when introduced in methylcholanthrene appears to be about 3 times as rapid as when it is injected in the form of dibenzanthracene.



EXTRACTION OF METHYLCHOLANTHRENE TUMORS

CHART 4

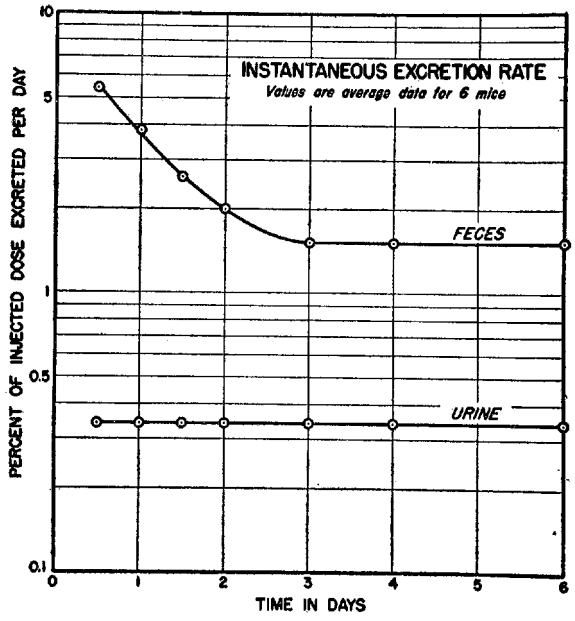


CHART 5.—Instantaneous excretion rate

ACKNOWLEDGMENTS

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Enzyme Alterations Associated with Mouse Liver Degeneration and Regeneration after Single Carbon Tetrachloride Feeding*

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Information on the role of enzymes in various types of normal and abnormal growth is essential for a better understanding of the vital activities of cells. As an extension of the chemical characterization of the degenerative and regenerative processes induced in mouse liver by carbon tetrachloride feeding (21), some enzymatic alterations associated with these processes were studied. The succinoxidase and cytochrome oxidase systems, as well as liver esterase and acid and alkaline phosphomonoesterases, were investigated.

The plan of the present study was similar to that previously described (21). Groups of mice were sacrificed at various intervals following carbon tetrachloride feeding until all degenerative and regenerative changes appeared to be complete. The present enzymatic investigation was conducted in close correlation with a general chemical and histological characterization of the various tissues utilized.

MATERIALS AND METHODS

Male strain A mice 2–3 months of age, weighing 22 ± 1 gm., were selected for these investigations. Each mouse was fed by trocar 0.1 ml. of a 40 per cent solution of carbon tetrachloride in olive oil (2), following which procedure groups of mice were sacrificed after 1, 2, 3, 4, 5, 6, 12, and 18 days. All mice underwent an 18-hour fast period prior to sacrifice. At the specified intervals following carbon tetrachloride feeding, the livers were removed from the mice under ether anesthesia. Each liver was weighed and homogenized separately in an all-glass homogenizer (Scientific Glass Co.) with a volume of redistilled water equivalent to 9 ml. per gram of liver. In special experiments utilizing differential centrifugation technics for the separation of cellular constituents, 30 per cent sucrose solutions were substituted for the water as the homogenizing medium. Representative tissues were taken for histological fixation prior to homogenization. The prepared homogenates were used directly for the enzymatic analyses as well as for several pertinent chemical measurements. All tissues were maintained as close to 0° C. as possible until used.

Although detailed chemical (21) and histological (19) descriptions of the changes induced in mouse liver following a single feeding of carbon tetrachloride have been previously presented, pertinent measurements were repeated because of some variation in response to carbon tetrachloride observed between groups of mice. Each liver sample utilized for the enzymatic investigations was characterized with respect to weight and content of lipid and moisture by procedures previously described (21).

The tissues were fixed in Stieve fluid and stained with hematoxylin and eosin. The sections were examined for necrosis and mitotic cells for each of the selected time intervals. The extent of damage was determined for the various liver samples by procedures originally proposed by Chalkley (1) for the quantitative estimation of tissue components. Measurements were made of viable liver cell cytoplasm, as determined by the retention of normal staining characteristics, as an indirect estimation of the extent of liver damage for each of the time intervals investigated.

Aerobic succinoxidase and cytochrome oxidase activities were determined manometrically by the homogenate technics as described by Schneider and Potter (17). All measurements were made at 37° C. with standard Warburg equipment. Following the first 10 minutes allowed for equilibration, assays were conducted over a 40-minute period, with readings taken at 10-minute intervals; little loss in activity for either enzyme was found within this time. Endogenous oxygen uptake was

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found to be negligible at the tissue concentrations used. Succinoxidase assays were conducted on at least two concentrations of tissue. Ascorbic acid was used as the reductant in the cytochrome oxidase assay with suitable corrections introduced for the autoxidation of this compound. Cytochrome oxidase assays were performed on five individual liver samples for each time interval investigated. Succinoxidase assays were performed on only three of the five liver samples utilized for the cytochrome oxidase study.

Esterase levels were also determined manometrically with slight modifications of published procedures (14, 16). Tributyrin was utilized as the substrate. Concentrations of tributyrin of between 0.75 and 3.0 per cent were found to be optimal, with little influence of the substrate at these levels upon enzymatic activity over a considerable period of time. All assays were conducted at two levels of tissue concentration. Concentrations of bicarbonate ion and CO_2 gas were selected to maintain the reaction mixture at approximately pH 7.4. Readings were taken for a total of 40 minutes at intervals of 10 minutes.

A detailed description of methods and characterization studies of the phosphatases of mouse liver will be presented elsewhere.[1] Phosphomonoesterase activity was determined at pH 5.5 and 9.2 with disodium phenyl phosphate as the substrate. Methods were developed to insure reliable quantitative measures of enzymatic activity at each of these levels of pH. The methods adopted for routine analyses will be described.

The reaction mixtures for acid and alkaline phosphomonoesterase assays were prepared to contain 0.5 ml. of 0.5 M disodium phenyl phosphate, 0.5 ml. of 0.75 M Mg ions, 1.5 ml. of 0.1 M buffer (acetate buffer at pH 5.5 for the acid and veronal buffer at pH 9.2 for the alkaline assay), and 0.5 ml. of liver homogenate, making a final volume of 3.0 ml. All assays were conducted on two levels of tissue concentration with between 0.1 and 1.0 per cent liver homogenates for the acid phosphomonoesterase and 2–3 times more concentrated tissue homogenates for the alkaline enzyme. All reactions were carried out in 25-ml. Erlenmeyer flasks at 37° C. for a 1-hour period in a constant temperature water bath, with specially constructed shaking racks to insure adequate mixing throughout the entire incubation period. Following incubation, 7 ml. of 5 per cent trichloroacetic acid solution was pipetted into the reaction vessels and the contents filtered using Whatman No. 5 paper. Aliquots of the filtrate were removed for the determination of inorganic phosphorus by the method of Fiske and Subbarow (3). Appropriate tissue and substrate blanks were also prepared for each of the samples investigated. It was necessary to purify further the commercial preparations of disodium phenyl phosphate from contaminating decomposition products prior to their use by treating with calcium to remove free phosphate and with alcohol to remove free phenol. By adopting the described procedures, straight-line relationships between tissue concentration and enzymatic activity were obtained over a wide range of tissue level. Acid phosphomonoesterase was found to be extremely labile,[1] necessitating a standardization of the time interval between liver removal and enzymatic assay. A standard 1-hour interval was adopted for each of the tissues investigated.

[1] Observations to be published from this laboratory.

RESULTS

The minor variations in response to the effects of carbon tetrachloride observed in different series of mice necessitated a general characterization of the liver samples utilized for the present investigation. The results of this study have been summarized in Chart 1. Following carbon tetrachloride feeding, a marked enlargement of the liver occurs, reaching a maximum after approximately 3 days. The increases in liver size could not be attributed to changes in the water or lipid content of this organ. Measurements for moisture indicated some accumulation within the livers; however, corresponding losses in the level of lipid were found to be associated with these increases. An interesting relationship exists between liver lipid and moisture subsequent to carbon tetrachloride feeding in that an increase in the one constituent is accompanied by a corresponding decrease in the other, resulting in little net change in the total level of both at any given time. The increases in liver weights found subsequent to carbon tetrachloride feeding cannot be attributed, therefore, to these constituents. Cytological measurements for liver damage revealed a maximum reduction in viable liver cell cytoplasm on the second and third days following carbon tetrachloride feeding. At these times viable liver cell cytoplasm was reduced per unit volume of liver to less than one-half of that found for the controls. The extent of liver damage was estimated in terms of remaining viable liver cell cytoplasm for purposes of subsequent comparisons with data obtained on enzymes known to be associated primarily with cytoplasmic structures. Regenerative changes were apparent from the first day with some mitotic cells present at this time. Maximum mitotic activity appeared to be occurring on the second day, with infrequent mitosis subsequent

to the third day. From these observations it is evident that a considerable overlapping between the degenerative and regenerative processes is present. The increases observed in liver weights

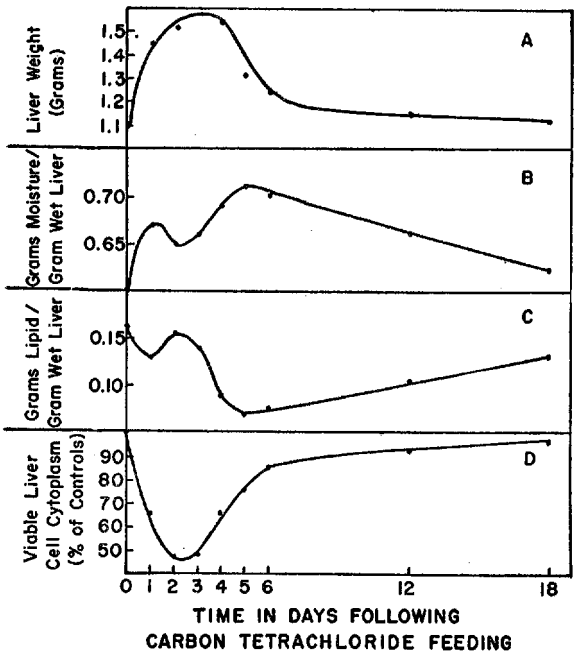

CHART 1.—Alterations induced in the weight (*A*), content of moisture (*B*), and lipid (*C*), and the proportion of viable cytoplasm present (*D*) in mouse liver following a single feeding of carbon tetrachloride. Separate analyses were made on five individual liver samples for each time interval investigated.

subsequent to carbon tetrachloride feeding were concluded, therefore, to be due to a net increase in liver bulk, resulting from regenerative growth proceeding at a rate in excess of the removal of necrotic cells.

Succinoxidase and cytochrome oxidase.—The results of the investigation of oxidative enzymes in relation to liver degeneration and regeneration in mice have been summarized in Table 1. In view of marked alterations in the lipid and moisture content as well as size of mouse liver subsequent to carbon tetrachloride feeding (Chart 1), the results of the enzymatic analyses were computed on the basis of activity per unit lipid-free dry liver as well as for total liver. By adopting these two measures, relative changes in enzyme levels as well as absolute increases or decreases could be computed.

Succinoxidase activity per unit lipid-free dry liver was found to be substantially reduced through the first 4 days subsequent to carbon tetrachloride feeding. In view of an accumulation in lipid-free dry liver mass within the first 4 days, loss in enzyme in terms of total liver was less. The increases in liver mass within this period were previously concluded to be due to regenerative growth proceeding at a rate in excess of the removal of necrotic cells. By the fifth day, a sharp increase in succinoxidase was found approximating normal values per unit lipid-free dry liver and greatly exceeding normal values for the total liver. On the sixth day, by which time little evidence of degenerative changes remains, more enzyme was present per unit lipid-free dry liver as well as per total liver. A gradual reduction approaching normal levels of succinoxidase was found subsequent to the sixth day.

The results of the cytochrome oxidase study were quite different from those described for succinoxidase. A marked reduction of this enzyme was found in the liver subsequent to carbon tetrachloride administration, followed by a gradual restoration resulting in somewhat less than normal levels after 18 days. Resynthesis of this enzyme in relation to general liver restoration proceeds at a more uniform and much less rapid rate than that observed for succinoxidase.

To evaluate further the results of the enzymatic study, comparisons were made between the levels of succinoxidase and cytochrome oxidase and the proportion of viable liver cytoplasm present for each of the time intervals investigated (Chart 2).

TABLE 1

SUCCINOXIDASE AND CYTOCHROME OXIDASE ACTIVITY IN MOUSE LIVER FOLLOWING CARBON TETRACHLORIDE FEEDING

No. of days following carbon tetrachloride feeding	Succinoxidase activity* Q_{O_2} (Lipid-free)†	Succinoxidase activity* $\mu l\ O_2$/hour/total liver at 37° C. ($\times 10^3$)	Cytochrome oxidase activity* Q_{O_2} (Lipid-free)†	Cytochrome oxidase activity* $\mu l\ O_2$/hour/total liver at 37° C. ($\times 10^3$)
Controls	88 (73–97)	20 (18–26)	330 (270–450)	90 (74–110)
1	77 (72–80)	18 (16–22)	200 (180–220)	55 (54–59)
2	62 (51–80)	16 (12–21)	200 (150–220)	58 (38–67)
3	55 (36–66)	18 (13–24)	160 (120–200)	54 (46–68)
4	56 (54–58)	17 (15–19)	210 (170–250)	73 (57–79)
5	93 (79–118)	29 (20–33)	220 (160–260)	66 (40–82)
6	109 (78–117)	30 (25–34)	230 (160–270)	69 (61–72)
12	92 (91–94)	26 (24–27)	270 (220–320)	75 (54–96)
18			280 (220–360)	79 (54–96)

*Succinoxidase assays were conducted on three and cytochrome oxidase assays on five individual liver samples for each time interval investigated. Control values represent averages of analyses on ten separate liver samples.

†Refer to μl. O_2 consumed per hour per milligram lipid-free dry liver at 37° C.

Viable liver cell cytoplasm was selected in preference to total viable liver in view of the recognized localization of these enzymes within cytoplasmic granules (9, 10). Enzyme levels were computed

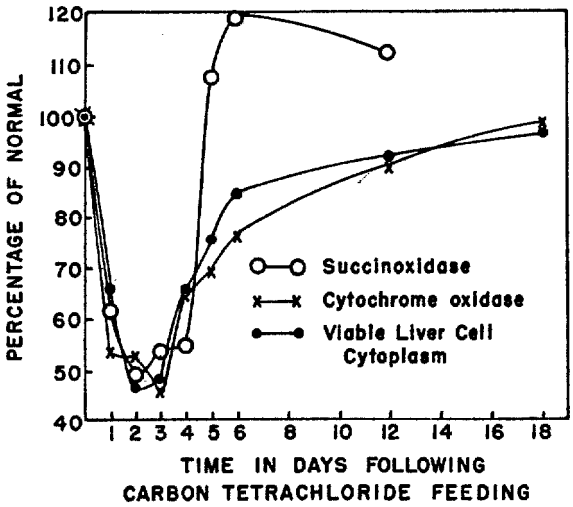

CHART 2.—Comparison between succinoxidase and cytochrome oxidase levels and the proportion of viable liver cell cytoplasm estimated to be present following a single feeding of carbon tetrachloride.

a remarkably good correlation. These findings suggest the presence of a normal average quantity of this enzyme within the viable liver cells at all times during the regenerative process. Succinoxidase activity, on the other hand, shows only an initial correlation; however, subsequent to the fourth day, a sharp increase in the level of this enzyme was found in relation to the proportion of nonnecrotic cytoplasm present. A marked resynthesis of succinoxidase occurred between the fourth and fifth days, resulting in approximately a twofold increase in enzyme, whereas only slight increases in viable liver cell cytoplasm were observed during this period of time. Little mitotic activity was observed after the third day, suggesting that resynthesis of this enzyme occurs at a time subsequent to cellular division.

The enzymes succinoxidase and cytochrome oxidase have been considered to be associated with sedimentable cytoplasmic granules identified cytologically as mitochondria (10). Special experiments were performed to isolate and characterize these cellular elements with respect to their content of succinoxidase and cytochrome oxidase on the third, fourth, and fifth days following carbon tetrachloride feeding. The procedures utilized for

TABLE 2

SUCCINOXIDASE AND CYTOCHROME OXIDASE ACTIVITIES IN ISOLATED LARGE GRANULE FRACTIONS (MITOCHONDRIA) FROM MOUSE LIVER FOLLOWING CARBON TETRACHLORIDE FEEDING

No. days following carbon tetrachloride feeding	Succinoxidase activity*: Total liver homogenate: μl O_2/hr/gm wet liver (×10³)	Succinoxidase activity*: Total liver homogenate: $Q_{O_2}N$‡	Succinoxidase activity*: Mitochondria fraction†: Total μl O_2/hr/gm wet liver (×10³)	Succinoxidase activity*: Mitochondria fraction†: $Q_{O_2}N$‡	Cytochrome oxidase activity*: Total liver homogenate: μl O_2/hr/gm wet liver (×10³)	Cytochrome oxidase activity*: Total liver homogenate: $Q_{O_2}N$‡	Cytochrome oxidase activity*: Mitochondria fraction†: Total μl O_2/hr/gm wet liver (×10³)	Cytochrome oxidase activity*: Mitochondria fraction†: $Q_{O_2}N$‡
Controls	21.6 (18.1–25.7)	610 (540–680)	9.7 (7.5–11.5)	1,300 (1,020–1,470)	99.0 (81.0–110)	2,770 (2,390–3,140)	51.4 (46.1–55.1)	6,900 (6,300–7,500)
3	12.3 (8.4–15.5)	430 (330–490)	5.3 (3.2–8.0)	1,090 (840–1,400)	36.4 (35.4–37.3)	1,290 (1,180–1,400)	21.1 (14.3–30.7)	4,370 (3,400–5,400)
4	10.6 (8.40–14.8)	360 (270–480)	5.8 (4.3–8.1)	1,250 (900–1,500)	44.5 (40.3–51.3)	1,530 (1,340–1,680)	25.7 (19.7–33.9)	5,500 (4,700–6,200)
5	22.9 (21.1–25.8)	660 (600–740)	10.4 (10.4–10.5)	1,540 (1,500–1,600)	69.1 (64.0–75.0)	1,980 (1,910–2,090)	46.0 (36.7–50.8)	6,800 (5,260–7,800)

* Four separate liver samples prepared from individual animals were assayed for each time interval including the controls.

† The mitochondria fractions were assayed for enzyme directly without washing.

‡ Refers to μl. O_2 consumed per hour per milligram nitrogen at 37° C. Nitrogen was determined by the micro-Kjeldahl method of Pregl (15).

on the basis of activity per unit wet weight of tissue. This unit was considered the most applicable for comparative purposes with the volume measurements for viable liver cell cytoplasm. The cytological measurements represent average values found on three individual liver samples for each of the time intervals.

A comparison of the cytochrome oxidase levels with the proportion of viable liver cell cytoplasm present for each of the time intervals demonstrates the isolation of this cellular fraction were the same as described by Hogeboom, Schneider, and Pallade (10).

The results of the centrifugation study have been summarized in Table 2. The third, fourth, and fifth day intervals were selected for this study in order to investigate further the marked increases in succinoxidase observed within this period of time. The results of enzymatic analyses on the total liver homogenates were essentially

similar for this and the previous series of mice presented in Table 1. Analyses for succinoxidase activity on the isolated mitochondria showed that only about half of the original total enzyme had been recovered in these fractions from the experimental as well as control tissues. Succinoxidase activity per unit of nitrogen in the isolated mitochondrial fractions was found to be less than the controls on the third and fourth days, with increases above control values recorded on the fifth day. The extent of contamination of the mitochondria fractions by necrotic tissue elements could not be determined. The fractionation studies indicated that the sharp increases observed in succinoxidase levels in the total liver homogenates between the fourth and fifth days could be attributed to a marked resynthesis of mitochondria containing somewhat more than normal levels of enzyme within this period.

Cytochrome oxidase activity per unit of isolated nitrogen in the recovered mitochondria fractions was less on the third and fourth days and approximately equal to the control values by the fifth day. However, as previously found (Table 1), less than normal levels of this enzyme were present per unit of liver tissue even after 5 days. By the fifth day, therefore, the mitochondria present appear to be normal with respect to their content of cytochrome oxidase; however, somewhat less than normal quantities of mitochondria appear to have been synthesized at this time.

Liver esterase.—Following a single feeding of carbon tetrachloride, marked alterations were observed in the levels of esterase present in mouse liver (Table 3). For purposes of comparison, the results were computed on the basis of wet weight, lipid-free dry weight, nitrogen, and total liver. With the first three adopted units, fairly comparable changes were shown in enzyme levels between the various time intervals followed, as would perhaps be expected from the characterization studies presented in Chart 1. Changes in the absolute level of liver esterase could also be determined by computing the results on the basis of activity per unit total liver.

The maximum reduction in liver esterase on the second day after carbon tetrachloride administration was followed by a rapid restoration resulting in an appreciable increase in enzyme above control values by the fourth day. As compared to the control series, more enzyme per unit of liver tissue, as well as an increased level of enzyme per unit total liver, was recorded for the fourth day. These findings would suggest the presence of somewhat more enzyme per cell and an over-all increase in the number of cells at this time interval. Following maximum accumulation, a gradual reduction occurred subsequent to the fourth day, resulting in normal levels of enzyme by the eighteenth day.

Acid and alkaline phosphomonoesterase.—The alterations induced in the levels of acid and alkaline phosphomonoesterases in relation to liver degeneration and regeneration have been summarized in Table 4. A marked decrease was observed in the level of acid phosphomonoesterase throughout the first 3 days subsequent to carbon tetrachloride feeding. These decreases correlated well through the third day with the estimated reduc-

TABLE 3

ESTERASE ACTIVITY IN MOUSE LIVER FOLLOWING CARBON TETRACHLORIDE FEEDING

No. of days following carbon tetrachloride feeding	Esterase activity* Per gram wet liver ($\times 10^3$)	Per milligram lipid-free dry liver ($\times 10^3$)	Per milligram nitrogen ($\times 10^3$)	Per unit total liver ($\times 10^3$)
Controls	470 (410–500)	2.1 (1.8–2.4)	13.9 (12.4–15.3)	570 (510–600)
1	240 (190–300)	1.2 (0.97–1.6)	7.8 (6.1–9.9)	350 (270–410)
2	120 (79–160)	0.62 (0.39–0.85)	4.6 (3.1–6.1)	180 (130–250)
3	160 (90–290)	0.79 (0.45–1.2)	5.8 (3.5–8.8)	260 (140–480)
4	560 (530–620)	2.5 (2.3–2.7)	17.0 (15.8–19.0)	860 (750–1,000)
5	450 (280–610)	1.9 (1.1–2.5)	12.7 (8.0–16.0)	590 (280–770)
6	550 (470–650)	2.3 (1.8–2.6)	15.0 (12.6–17.0)	680 (510–790)
12	580 (490–690)	2.4 (2.2–2.7)	15.7 (14.5–17.3)	670 (570–790)
18	510 (420–590)	2.0 (1.8–2.4)	13.7 (12.3–15.4)	560 (510–630)

* Expressed in μl. CO_2 evolved per hour at 37° C. from a bicarbonate buffer (pH 7.4). Assays were conducted on five separate individual liver samples for each time interval investigated except the control series, which represents analyses on ten separate normal liver samples.

tions found in viable liver cell cytoplasm (Chart 1, *D*). Subsequent to this time, a sharp increase in the level of this enzyme was noted, resulting in an approximately twofold accumulation of enzyme within a 24-hour period. By the fourth day, therefore, approximately normal levels of enzyme were present per unit of lipid-free dry liver, with appreciably more than normal levels observed for the total liver. It might be concluded that there is more enzyme per cell at this time, as compared to the control tissues, in view of appreciable quantities of contaminating degenerative tissue elements, containing presumably little if any remaining enzyme (Chart 1, *D*).

The results of the alkaline phosphomonoesterase study were quite different from those observed for the acid series. Alkaline phosphomonoesterase activity was only slightly reduced below

normal levels at a time when maximum liver destruction was observed (compare with Chart 1, *D*). Subsequent to the initial slight decreases, a gradual accumulation of this enzyme occurred, resulting in appreciably greater than normal levels by the third day and reaching maximum values on the fifth day. Approximately normal levels of enzyme were not approached until the eighteenth day.

TABLE 4

ALTERATIONS INDUCED IN ACID AND ALKALINE PHOSPHOMONOESTERASE ACTIVITIES DURING LIVER DEGENERATION AND SUBSEQUENT REGENERATION

No. of days following carbon tetrachloride feeding	Acid phosphomonoesterase activity*		Alkaline phosphomonoesterase activity*	
	Per gram lipid-free dry liver	Per unit total liver	Per gram lipid-free dry liver	Per unit total liver
Controls	103 (89–124)	28 (24–32)	18 (16–19)	5.0 (4.5–5.6)
1	74 (68–85)	21 (19–23)	18 (15–22)	5.0 (4.3–5.8)
2	58 (53–62)	17 (14–19)	16 (12–23)	4.7 (3.6–6.7)
3	60 (50–70)	19 (15–27)	23 (16–27)	7.3 (5.4–11.0)
4	104 (93–110)	36 (30–42)	28 (21–31)	9.5 (6.7–11.0)
5	91 (80–99)	28 (20–34)	31 (21–38)	9.6 (5.1–13.1)
6	98 (91–102)	30 (24–35)	25 (20–31)	7.5 (5.2–12.2)
12	100 (86–109)	28 (23–33)	21 (20–22)	5.7 (5.2–6.6)
18	111 (92–123)	31 (26–34)	19 (16–25)	5.4 (4.1–7.6)

* Reported in mg. P released per hour at 37° C. determined at pH 5.5 and 9.2 for acid and alkaline phosphomonoesterase, repectively. All assays were conducted on five separate individual livers for each time interval investigated except the control series, which represent analyses on ten separate normal livers.

DISCUSSION

There is not complete agreement on the mode of action of carbon tetrachloride on liver cells. The concept that it produces a swelling of the liver parenchyma resulting in ischemia of the central areas of the lobule and ultimate death of the affected cells from oxygen deprivation (8) has not been supported by some other evidence (19). Whatever may be the action of the toxic substance, an over-all reduction in the level of total liver enzymes occurs as these cells die. The decreases in enzyme levels approximate the estimated reductions observed in total viable liver cell cytoplasm in the cases of succinoxidase, cytochrome oxidase, and acid phosphomonoesterase. However, other enzymes, such as liver esterase, appear to be more susceptible than the liver cells themselves to the toxic effects produced by carbon tetrachloride so that a greater reduction in their levels is found in proportion to the number of dead cells present. On the other hand, enzymes such as alkaline phosphomonoesterase appear to be but slightly reduced within the total liver in spite of the presence of extensive cellular destruction. Little agreement appears to be present among various investigations (7, 12, 19, 22) regarding the relative presence or absence of alkaline phosphomonoesterase within necrotic liver cells as determined by histochemical staining technics (4). However, an absence of this enzyme was observed within the necrotic liver cells of mice soon after carbon tetrachloride feeding in experiments performed in this laboratory (19). Assuming an absence of this enzyme within the necrotic liver areas, a rapid parallel resynthesis by the remaining viable liver cells or else a possible disturbance in the excretion of the enzyme from the liver might be suggested as an explanation of the experimental results.

Active liver regeneration occurs soon after carbon tetrachloride administration, with the first mitotic cells present at 24 hours. Increases in enzymes were found subsequent to maximum mitosis, indicating that resynthesis of new enzyme occurs presumably following cellular division. It is of some interest to compare the relative rates at which the various enzymes are restored within the newly formed liver cells. Increases in liver esterase and acid phosphomonoesterase were observed between the third and fourth days. Liver esterase appears to be localized to a great extent (14) and acid phosphomonoesterase to some extent[1] within the submicroscopic sedimentable fractions (microsomes) obtained by centrifugation technics. Increases in these enzymes might conceivably be indicative, therefore, of resynthesis of microsomes during this period within the newly formed liver cells. However, centrifugation experiments were not performed to test this possibility. Increases in succinoxidase levels were not apparent until the fifth day. The increases in enzyme observed at this time were found to be associated with a considerable increase in the amounts of material isolated in the mitochondria fractions. Synthesis of new mitochondria as well as enzyme within the young liver cells presumably occurs, therefore, within this interval. In contrast to the rapid resynthesis of succinoxidase, cytochrome oxidase is restored within the newly formed liver cells at an even rate over a prolonged period of time.

The resynthesis of alkaline phosphomonoesterase in relation to cellular growth remains confusing. Increases in this enzyme were greater than those of the other enzymes investigated. Little difference in the levels of alkaline phosphomonoesterase has been reported (6) between normal adult and regenerating rat hepatic tissues. On the

other hand, Sulkin and Gardner (20), using histochemical technics, have found increased levels of this enzyme within regenerating rat liver and ascribe some significance to these findings with respect to the restoration process. In view of the recognized importance of the liver in excreting alkaline phosphomonoesterase into the bile (7, 12) it would appear hazardous to attach too much significance to increases in this enzyme where hepatic injury might also be suspected. The increased levels of alkaline phosphomonoesterase observed in the present investigation might be attributed to a disturbed excretion rather than to an increased synthesis of new enzyme.

Slight increases in succinoxidase as well as in liver esterase were found to be associated with liver restoration following carbon tetrachloride feeding. Novikoff and Potter (13) reported a decreased level of succinoxidase in regenerating rat liver following partial hepatectomy when computing their results on a basis of dry tissue weight (Q_{O_2}). However, perhaps the apparent decreases could be attributed to some extent to the presence of abnormally large amounts of lipids present in regenerating liver tissues following partial extirpation.[1] Neoplastic and fetal liver tissues have been reported to contain less esterase and regenerating liver tissue contains approximately the same level of this enzyme as normal adult resting liver (5). The presence of a decreased level of liver esterase in association with liver damage following the administration of other hepatotoxic agents (phosphorus and chloroform) has been previously reported by others (11). Cytochrome oxidase levels subsequent to carbon tetrachloride feeding were found to approximate the proportion of viable liver cell cytoplasm measurements for each of the time intervals investigated. This is not in disagreement with Shack (18), who found little difference in cytochrome oxidase levels between 48-hour regenerating and normal rat liver when compared on a basis of wet tissue weight.

SUMMARY AND CONCLUSIONS

Following a single feeding of 0.1 cc. of 40 per cent carbon tetrachloride to strain A male mice, the changes in histological characteristics and content of succinoxidase, cytochrome oxidase, liver esterase, and acid and alkaline phosphomonoesterases of the liver during the resulting degenerative and regenerative processes have been studied and evaluated. Under the conditions of this experiment the liver necrosis, as evidenced by the reduction in viable liver cell cytoplasm, starts during the first day and reaches a maximum during the second to third days after feeding carbon tetrachloride. The tissue repair as evidenced by mitotic activity started after the first day, so that the processes of liver degeneration and regeneration proceeded concomitantly.

The destruction of liver tissue was accompanied by a reduction in all the enzymes studied. Losses in succinoxidase, cytochrome oxidase, and acid phosphomonoesterase approximated the estimated proportion of liver cell cytoplasm destroyed while alkaline phosphomonoesterase showed a lesser and liver esterase a greater extent of reduction. Subsequent to maximum mitotic activity resynthesis of new enzyme occurred. Acid phosphomonoesterase and liver esterase were restored within the new liver cells prior to maximum restoration of succinoxidase and cytochrome oxidase. All enzymes investigated except cytochrome oxidase were resynthesized within comparatively short periods of time after maximum reductions in their levels were noted.

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The Nucleic Acid Inhibiting Action of 4-Amino-N^{10}-Methylpteroylglutamic Acid in Mice with a Sensitive and Resistant Strain of Leukemia*

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It is well established that folic acid antagonists provide temporary palliation in certain cases of acute leukemia in children (7) and that several compounds of this class increase the survival time of mice with transplanted leukemia Ak 4 (4, 10, 11). However, eventual failure in the treatment of leukemia by chemotherapy with such compounds occurs consistently.

The mechanism involved in the development of drug resistance in leukemia is at present poorly understood. The lack of fundamental knowledge on this point makes it difficult to utilize the known anti-leukemic agents to the fullest advantage.

It has been recently demonstrated that a hitherto sensitive strain of leukemia may be rendered resistant to 4-amino-N^{10}-methylpteroylglutamic acid (A-methopterin) by repeated passage through treated mice (5). This strain shows a cross resistance to five other 4-amino antagonists of pteroylglutamic acid (2). These observations may parallel the ultimate failure of folic acid antagonists in the treatment of patients with acute leukemia.

Law and Boyle (9) have reported the development of resistance in three separate sublines of a transplantable lymphoid leukemia in dba mice following successive transplants in mice treated with three different folic acid antagonists. These refractory strains carried over resistance from one antagonist to another. One of us has demonstrated that a strain of mice made resistant to A-methopterin retained its sensitivity to the anti-leukemic effects of a crude antagonist of pteroylglutamic acid (3) and 2,6-diaminopurine (6).

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Recent findings concerning the effect of folic acid antagonists on nucleic acid synthesis suggest that folic acid or some metabolite of this compound may be acting as the prosthetic group of an enzyme necessary for purine synthesis (8, 13). Certainly, A-methopterin or 4-aminopteroylglutamic acid (aminopterin) exerts a profound inhibitory effect on nucleic acid synthesis *in vivo* (13).

The following experiments were conducted with the thought in mind that the failure of the resistant strain of leukemia to respond to A-methopterin might be due to a failure of this compound to inhibit sufficiently nucleic acid synthesis in the refractory leukemic cells.

EXPERIMENTAL

The 4-amino-N^{10}-methylpteroylglutamic acid resistant subline of mouse leukemia (Ak 4-R) used in these studies has already been described (5). This drug-fast strain showed no significant response to treatment with A-methopterin, while the original strain (Ak 4) responds to the extent that one observes a consistent increase in life span, in treated compared to untreated mice, of greater than 100 per cent.

The method used to determine the rate of synthesis of nucleic acids and nucleic acid purines has also been reported (13). This technic consisted of injection of carbon 14-labeled sodium formate (a rather specific precursor of the 2- and 8-carbon atoms of the nucleic acid purine skeleton [1]) and isolation of the viscera nucleic acids and nucleic acid purines after a period of 6 hours. Activity assays on these fractions provide data which can be used to estimate the rate of biosynthesis of nucleic acid purines and, in turn, the larger nucleic acid polymers. As has been mentioned, strong folic acid antagonists profoundly affect incorporation of formate carbon into these important cell fractions (13). We have also observed that x-radiation, 2,6-diaminopurine, cortisone,

and a combination of urethan plus nitrogen mustard significantly affect purine synthesis under conditions where over-all formate fixation into tissue is not reduced (14).

To compare the action of a given dose of A-methopterin on nucleic acid purine synthesis in the nonresistant (Ak 4) and the resistant (Ak 4-R) leukemic strains, experiments were carried out as follows: Groups of Akm mice were inoculated with Ak 4 and Ak 4-R leukemia, and, after the leukemia was well advanced (see blood counts, Table 1), the mice were in certain instances treated on the sixth and seventh, or sixth, seventh, and eighth days with A-methopterin. The level of injection was in all cases 3.0 mg/kg. On the seventh or eighth days after inoculation, the various groups were injected with 1.4 μc. of $HC^{14}OONa$ per mouse, and after 6 hours in a metabolism chamber the mice were sacrificed and the livers and spleens extirpated, pooled, and homogenized in a refrigerated Waring Blendor. Aliquots of this liver and spleen homogenate were then subjected to isolation procedures (14) which provided small quantities of combined nucleic acids and combined nucleic acid purines. These fractions were assayed for carbon 14 content by a gas phase procedure (12). The data obtained are summarized in Table 1.

TABLE 1

THE EFFECT OF A-METHOPTERIN ON NUCLEIC ACID SYNTHESIS IN MICE WITH AK 4 AND AK 4-R STRAINS OF LEUKEMIA

Exp. no.	Strains of leukemia	No. of mice	Days of treatment	Day of $HC^{14}OONa$ injection	WBC*	Specific activity (μc/mole carbon) Tissue homogenate†	Combined nucleic acids	Combined NA purines‡
1	Ak 4	7	none	7	42,500	4.56	90.0	242.1
2	Ak 4-R	10	none	7	38,500	6.45	34.1	236.6
3	Ak 4	10	6, 7	7	7,250	4.73	5.64	10.2
4	Ak 4-R	10	6, 7	7	21,500	5.29	11.83	64.8
5	Ak 4	10	6, 7, 8	8	8,800	5.59	7.82	17.5
6	Ak 4-R	9	6, 7, 8	8	30,000	8.18	23.10	50.2

NOTE: All experiments were of 6 hours' duration after intraperitoneal injection of 1.4 μc. of sodium formate.
* Average of two or three mice.
† Determined on an aliquot of the pooled liver-spleen homogenate.
‡ Twice precipitated as the silver salts.

DISCUSSION

It is of interest to note that A-methopterin has failed to inhibit nucleic acid synthesis in the livers and spleens of mice with a refractory strain of leukemia to the same degree as has been observed in a strain which responds to treatment with this drug. This observation has been repeated on a fairly large number of mice, and the differences are believed to be significant. Such results as have been observed might be attributed to the survival of a resistant mutant subline of leukemic cells adapted to therapy with 4-amino-N^{10}-methylpteroylglutamic acid. Several interpretations of the biochemical mechanisms involved in this adaptation might be considered:

a) The refractory strain of leukemia has a limited ability to detoxify A-methopterin. Since the anti-leukemic activity of this drug is based on preferential toxicity to the leukemic cell, a slight loss in this specificity would nullify the temporary effectiveness of this compound.

b) The Ak 4-R strain employs a different scheme for introduction of the single ureide carbon atoms into nucleic acid purines which is not so effectively blocked by A-methopterin. This possibility seems less likely, since it appears from the present data that the difference in formate fixation in the sensitive and resistant strains is but a relative one.

c) The resistant leukemia has acquired the ability to synthesize folic acid (or the citrovorum factor) and thus can reverse the anti-leukemic action of A-methopterin. A failure of A-methopterin plus sulfonamides (PABA antagonists) to increase the life span of mice with the resistant leukemia suggests that this mechanism is unlikely.[1] Also, microbiological assays of folic acid and the citrovorum factor have failed to show any significant difference between Ak 4 and Ak 4-R spleens or or livers.[2]

All the liver and spleen cells from which nucleic acids were isolated in this study were not, of course, leukemic. From previous studies, it can be estimated very roughly that at 8 days after inoculation of Ak 4 leukemia into Akm mice the liver almost doubles and the spleen more than triples in weight. This suggests that more than half the cells of the organs used in this study were made up of the respective leukemic strains. Had we been deal-

[1] J. H. Mitchell, Jr., and H. E. Skipper, unpublished data.

[2] J. H. Burchenal, E. Robinson, and A. Crossley, unpublished data.

ing with wholly neoplastic tissue, it seems likely that greater differences might have been observed.

Data presented in Table 1 on formate fixation in the nucleic aid purines from livers and spleens of leukemic mice, when compared to previously reported results obtained on viscera from nonleukemic animals (14), suggests that this disease is significantly accelerating nucleic acid synthesis.

Further studies on the effects of A-methopterin on formate fixation in desoxyribonucleic acid guanine, adenine, and thymine and ribonucleic acid guanine and adenine in the Ak 4 and Ak 4-R leukemias are planned.

SUMMARY

Nucleic acid synthesis in the livers and spleens of mice with a refractory strain of leukemia was not inhibited to the same extent by A-methopterin as was observed in mice with a leukemic strain which responds to treatment with this compound.

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Book Reviews

Index of Tumor Chemotherapy. By HELEN DYER. Bethesda: Federal Security Agency, Public Health Service, 1949.

The National Cancer Institute announces the publication of a new report entitled "An Index of Tumor Chemotherapy." The 329-page monograph compiled by Dr. Helen M. Dyer, biochemist, represents the most comprehensive survey made to date of the literature on the results of treatment of tumors by chemical methods. The work was undertaken in order to provide cancer research workers with a single source from which the available information on this subject could be obtained. The result is a compilation of data from the chief reports on tumor chemotherapy published in the American, English, French, Japanese, German, Italian, Portuguese, Scandinavian, and Spanish scientific literature.

The four main sections of the report are: (1) a historical résumé and explanatory introduction; (2) a table of 5,031 chemotherapeutic tests classified according to chemical used; (3) an alphabetical index of the chemicals, keyed so that they may be instantly located in the table of chemotherapeutic tests; and (4) a bibliography of the 2,213 references from which the data were taken. The table of chemotherapeutic tests contains, for each chemical, data on the type of tumor, species, and number of animals used, the dosage, number of treatments, route of administration, effect claimed, year the test was reported, and the reference.

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Requests for copies will be filled to the extent possible within the limitations imposed by the size of the edition and the relative needs of groups and individuals. Requests should be addressed to the Cancer Reports Section, National Cancer Institute, Bethesda 14, Maryland.

Lehrbuch der allgemeinen Pathologie und der Pathologischen Anatomie. By H. HAMPERL. Heidelberg: Springer-Verlag, 1950.

The Royal Cancer Hospital Mechanically Sorted Punch Card Index System. 2d ed. A. W. HUNT, D. W. SMITHERS, M. LEDERMAN, M. W. HARMER, and K. M. TURNER. Colchester, England: Benham & Co., Ltd., 1950.

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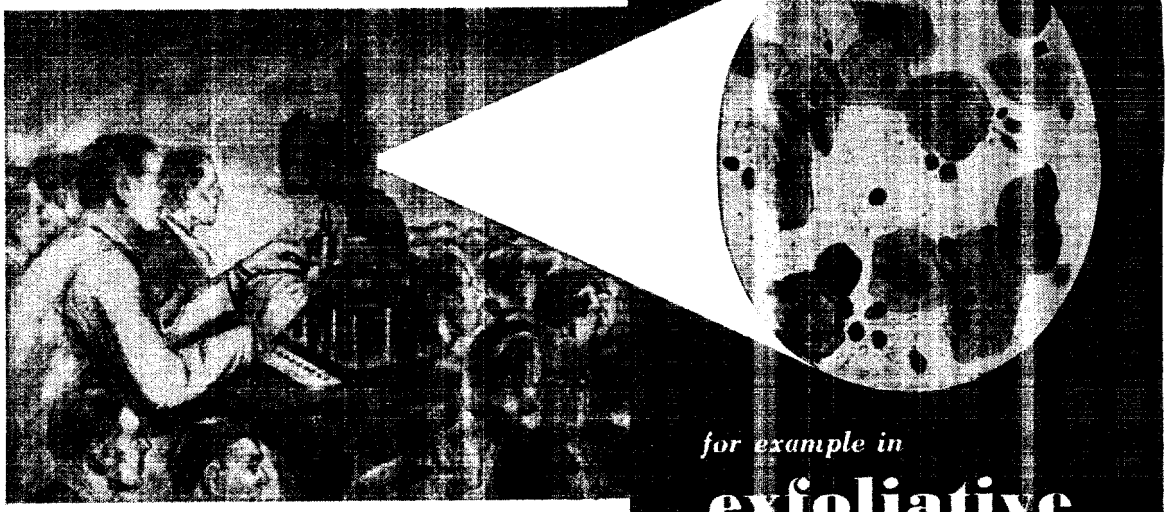

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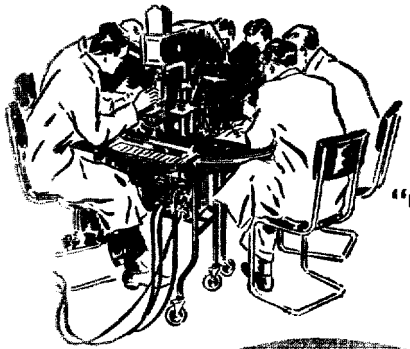

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